昭和32年7月9日 第三種郵便物認可
第45巻 第7号 昭和33年7月25日発行
毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 7, July 25, 1958

Vol. 45, No. 7 July, 1958

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI
SHIGERU AKAMATSU
SHICHIRO AKIYA
FUJIO EGAMI
SABURO FUNAHASHI
YOSOJI ITO
KOOZOO KAZIRO
KATASHI MAKINO
TOMOO MIWA
ZIRO NIKUNI
NORIO SHIMAZONO
YUSUKE SUMIKI
HIROSHI TAMIYA
ITARU WATANABE
HARUHISA YOSHIKAWA

PUBLISHED MONTHLY
BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Departments of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U. S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku Tokyo, Japan.

The Journal of Biochemistry, Vol. 45, No. 7, 1958

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

XIX. SUPPLEMENTARY STUDIES ON CHOLIC ACID DEGRADATION BY STREPTOMYCES RUBESCENS

BY SHOHEI HAYAKAWA*, YASUO SABURI**
AND HIDEYUKI HOSHIJIMA*

(From the Department of Biochemistry, Okayama University Medical School, Okayama, and** First Bacteriological Division, the Institute for Infectious Disease, the University of Tokyo, Tokyo)*

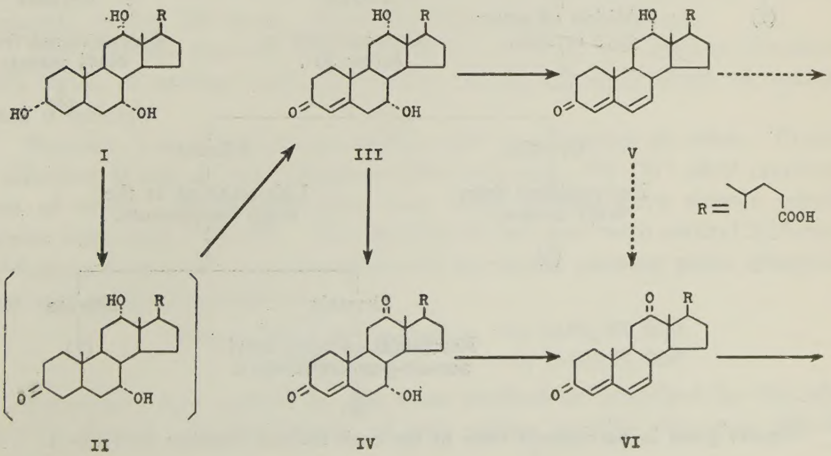
(Received for publication, January 8, 1958)

It is of interest that *Streptomyces rubescens* is capable of degrading cholic acid via an intermediate containing a Δ^4-ketone structure and a $\Delta^{4,6}$-3-ketone structure respectively. In a previous paper (*1*), the acids III and IV have been isolated as the intermediates containing a Δ^4-3-ketone structure, and the acid VI has been isolated as one containing a $\Delta^{4,6}$-3-ketone. In the same paper (*1*), a possible pathway for the cholic acid degradation by *S. rubescens* has been proposed and discussed on the basis of this result.

In the present experiment, a new degradation product of cholic acid by *S. rubescens*, 12α-hydroxy-3-oxo$\Delta^{4,6}$-choladienic acid (V), was isolated in addition to the two acids (IV and VI) out of the above three products (III, IV and VI).

DIAGRAM 1

A proposed pathway for cholic acid degradation by *S. rubescens*.



Now it is quite possible that the acid VI, isolated in a previous paper (*1*), may be derived from the acid IV or the acid V, and the acid V, isolated in this study, may be derived from the acid III as well as the formation of the acid VI from the adid IV.

Therefore a possible pathway for the cholic acid degradation by *S. rubescens* may be written as shown in Diagram 1. However, it is not yet clear whether the acid V is further degraded via an intermediate differed from the acid VI or not, and whether the acid V is oxidized to the acid VI or not.

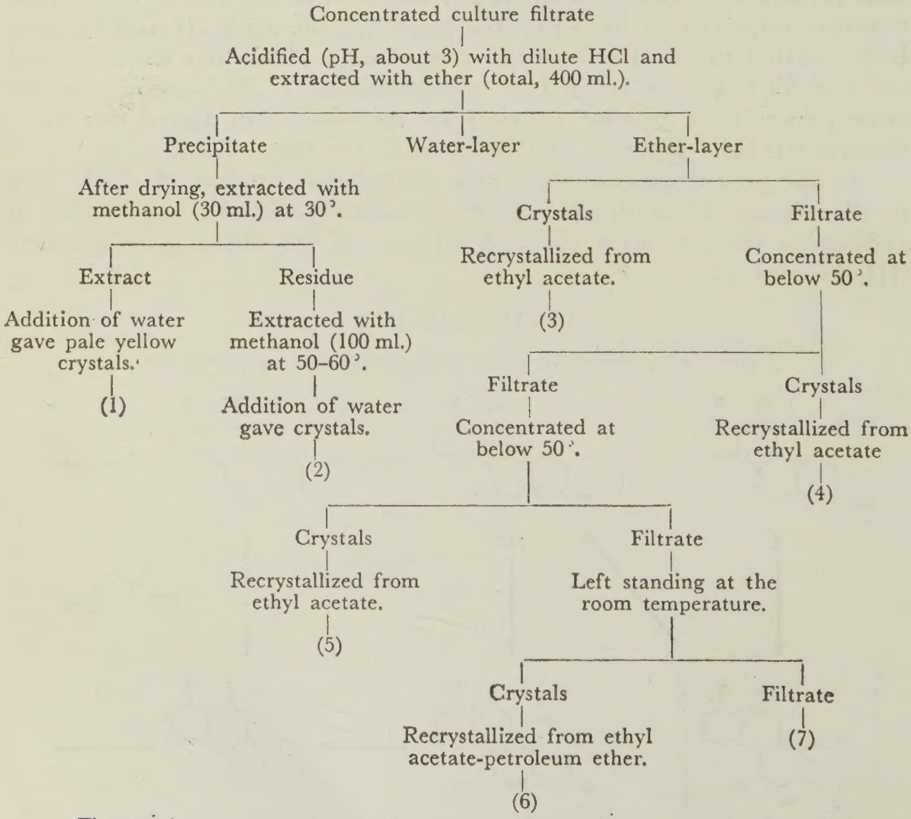
Furthermore it is quite likely that an appearence of a peak at 290 mμ in a cholate culture with *S. rubescence*, found and discussed in a previous paper (*1*), is due to the formation of both the acids V and VI.

EXPERIMENTAL

Cultivation and Isolationof Cholic Acid Metabolites—Sodium cholate (6 g.) was

SCHEME 1

Fractionation and Isolation of Cholic Acid Metabolites by S. rubescens


Concentrated culture filtrate
Acidified (pH, about 3) with dilute HCl and extracted with ether (total, 400 ml.).
Precipitate
Water-layer
Ether-layer
After drying, extracted with methanol (30 ml.) at 30°.
Extract
Residue
Addition of water gave pale yellow crystals.
(1)
Extracted with methanol (100 ml.) at 50–60°.
Addition of water gave crystals.
(2)
Crystals
Filtrate
Recrystallized from ethyl acetate.
(3)
Concentrated at below 50°.
Filtrate
Concentrated at below 50°.
Crystals
Recrystallized from ethyl acetate
(4)
Crystals
Recrystallized from ethyl acetate.
(5)
Filtrate
Left standing at the room temperature.
Crystals
Recrystallized from ethyl acetate-petroleum ether.
(6)
Filtrate
(7)

Figures given in parentheses refer to the each fraction number in Table I.

cultivated for 5 days with *S. rubescens* by a shaking culture and the degradation products were isolated by the same method as described in a previous paper (*1*). The outline and summary were shown in Scheme 1 and Table I.

TABLE I

Identification of Cholic Acid Metabolites Obtained from the Fractions in Scheme 1

Characteristics of each fraction in Fig. 2				Recrystallized from	m.p. ($^\circ C$)	Identified substances
No.	Yields (*mg.*)	m.p. ($^\circ C$)	$\lambda_{max}^{alc.}$ ($m\mu$)			
1	110	208–210	281	—	—	Unidentified**
2	50	235–242 (decomp.)	242 284	Methanol	240–243 (decomp.)	”
3	178	260–263 (decomp.)	241	Methanol-ethyl acetate	262–265 (decomp.)	”
4	60	248–251 (decomp.)	240	Methanol	248–250 (decomp.)	7α-Hydroxy-3,12-dioxo-Δ^4cholenic acid***
5	98	243–247 (decomp.)	240			
6	60	201–205	281	Ethyl acetate-petroleum ether	204–205.5	3,12-Dioxo-$\Delta^{4,6}$-choladienic acid****
7*	—	—	—	—	—	—

* See text for experimental details.

** A pure compound was not yet obtained.

*** *Analysis* Calcd. for $C_{24}H_{35}O_5$: C, 71.59; H, 8.51. Found: C, 71.61; H, 8. 59.

**** A satisfactory analytical data was not obtained. However the identity as this acid was confirmed by mixed m.p. test and infrared comparison.

Chromatographic Separation of Methyl 12α-Hydroxy-3-oxo-$\Delta^{4,6}$-choladienate and Methyl 3,12-Dioxo-$\Delta^{4,6}$-choladienate—Fraction No. 7 in Scheme 1 was esterified with diazomethane by the usual manner. The ester was dissolved in a small volume of ether and the ether solution was poured onto 15 g. of an active alumina (Wako, 200 mesh) column of dimensions 80×20 mm.

Five elutions (fraction 1–5) with 30 ml. of ether, one elution (fraction 6) with 30 ml. of acetone and one elution (fraction 7) with 30 ml. of methanol were obtained.

Fraction 1 and 5 gave no residue after evaporation of ether. Fraction 2 afforded 40 mg. of pale yellow needles with m.p. 172–175° after concentration of ether. Recrystallization from ethyl acetate gave almole colorless prisms with m.p. 174–176°. The identity of this ester with methyl 3,12-dioxo-Δ-^{4,6}-choladienate (*2*) was demonstrated by mixed melting point determination and infrared comparison.

Analysis	Calcd for $C_{25}H_{34}O_4$:	C, 75.34; H, 8.60
	Found:	C, 75.30; H, 8.48

Fraction 3 was treated by the same method as described for the above fraction 2 and afforded 30 mg. of pale yellow needles witn m.p. 183–184°.

Repeated recrystallizalion from methanol gave colorless prisms with m.p. 184–185.5°. The characterization of this ester as methyl 12α-hydroxy-3-oxo-$\Delta^{4,6}$-choladienate (*2*) was confirmed by mixed melting point determination and infrared comparison.

Analysis	Calcd. for $C_{25}H_{36}O_4$:	C, 74.96; H, 9.06
	Found:	C, 74.92; H. 9.02

Fraction 4,6 and 7 gave an oily mass after evaporation of the solvents, so these fractions were not subjected to the further treatments.

SUMMARY

In addition to the three degradation products (acids III, IV and VI) of cholic acid by *S. rubescens*, isolated in a previous paper (*1*), 12α-hydroxy-3-oxo-$\Delta^{4,6}$-choladienic acid (V) was isolated as its methyl ester in the present experiment.

Considering the sturctures of these intermediates, a degradation pathway of cholic acid, proposed in a pervious papee (*1*), was reproduced as seen in Scheme 1.

REFERENCES

(*1*) Hayakawa, S., Saburi, Y., and Tamaki, K., *J. Biochem.*, **45**, 419 (1958)
(*2*) Hayakawa, S., Saburi, Y., Fujii, T., and Sonoda, Y., *J. Biochem.*, **39**, 731 (1956)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

V. CONVERSION OF GLYOXYLIC ACID TO GLYCINE IN THE SILKWORM LARVA

BY TOSHIFUMI FUKUDA AND TOSHIO HAYASHI

(*From the Sericultural Experiment Station, Tokyo*)

(Received publication, January 28, 1958)

Biosynthesis of glycine by transamination of glyoxylic acid and amino acids has been recognized in the liver of rats by Meister *et al.* (*1*), in the intact rat by Weinhouse *et al.* (*2*), in tobacoo leaves by Wilson *et al.* (*3*) and in cell free extracts of a *Pseudomonas* by Campbell (*4*). Wright (*5*) clarified that glyoxylic acid could be used as effectivelly as glycine for the growth of a *Neurospora* mutant which specifically requires glycine. These observations seem to suggest that the transminase reaction involving glyoxylic acid is widespread in nature.

In the silkworm, Kirimura (*6*) concluded from a quantitative study that the glycine in the silkworm must be partly synthesized by this insect itself as its amount in mulberry leaves which were actually disgested by the insect does not account for the higher content of this amino acid in the cocoon fibres. Recently Koide *et al.* (*7*) clarified that glycine was synthesized by transaminase reaction between glyoxylic acid and amino acids in the alimentary canal of the silkworm. As a large quantity of glycine (33.6 per cent.) is contained in the silkproteins, the finding of precursors of glycine synthesis in the silkworm body seems to be of special importance in relation to the silk production of silkworm.

The present study was carried out to clarify whether glyoxylic acid is one of precursors of glycine synthesis in the silkworm. In the present study glyoxylic acid was isolated from the body fluid, and then it was elucidated that glycine was synthesized by transamination of glyoxylic acid and amino acids in the silkgland-cell of the silkworm.

EXPERIMENTAL

Isolation of Glyoxylic Acid from the Body Fluid—About 1.2 liter of the body fluid obtained from about 4,000 worms at the 3rd or the 4th day of the 5th instar, Si 108, were added to 20 liters of 70 per cent ethanol solution* of 2,4-dinitrophenylhydrazine in concentrated H_2SO_4 and the mixture were concentrated to about 500 ml. under reduced pressure. After standing a week, the mixture was filtrated. The precipitate was dissolved in ethyl acetate. The filtrate was adjusted to approximately pH 1 with 10 N sodium hydroxide and the hydrazones and hydrazine were extracted with ethyl acetate. The two ethyl acetate solutions were next extracted with successive portions of alkaline Na_2HPO_4 solution, first saturated, then $M/15$. The Na_2HPO_4 extracts were adjusted to approximately pH 1 with 10 N HCl, and the mixture of hydrazones and accompanying impurity of hydrazine were taken up once more in ethyl acetate. The solution of hydrazones in ethyl acetate was distilled off in vacuo at about 20°, leaving an oily orange residue. The residue was dissolved in saturated Na_2CO_3 and precipitated by the addition of 10 N HCl. The product was recrystallized from ethyl acetate and then from ether by the addition of light petroleum. It was dried in vacuo over $CaCl_2$ at room temperature. The yield was 8 mg.

---

* 1 g. of 2,4-dinitrophenylhydrazine was dissolved in 3 ml. of concentrated sulfuric acid and then the solution was added to 15 ml. of ethanol.

Transaminase Reaction Involving Glyoxylic Acid—a) Enzyme Preparations: The posterior division including silk-substances and the middle division except silk-substances of the silkglands obtained from one hundred worms at the 6th day of the 5th instar, Nichi 122 X Si 115, were respectively washed with cold water and suspended in Britton-Robinson's phosphate buffer, pH, 9.4 and homogenized. Each homogenate was placed for 5 hours at 0° and then kept frozen at −18° overnight. The insoluble proteins obtained by thawing the ice at room temperature were discarded and the supernatant solution was used as an enzyme solution for this experiment. *b) Substances*: Glyoxylic acid was prepared as the potassium salt by the method of Doebner (*8*) and its purity was ascertained according to the procedure described by Clift *et al.* (*9*). On the other hand, the purity of the following 14 amino acids was checked by paper chromatography: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, L-histidine, L-leucine, DL-serine, DL-threonine, L-tryptophan L-tyrosine and DL-valine. The occurrence of these amino acids in the silkworm body has already been elucidated, except cystine (*10*, *11*). The solution of glyoxylic acid and amino acids were, immediately prior to use, made in 0.05 M phosphate buffer, pH 9.4, at a concentration of 200 μM per ml. and 50 μM per ml. with respect to the L-form. *c) Incubation Conditions*: To 1 ml. of each enzyme solution were added 0.5 ml. of the glyoxylic acid solution and 1 ml. of the amino acid solution, and this mixture was incubated for 60 minutes at 38°. The reaction was stopped by adding 200 mg. of trichloracetic acid. The filtrate was used for the determination of glycine formed. *d) Determination of Glycine*: The concentration of the glycine formed in the reaction mixture was estimated with the procedure described by Giri (*12*, *13*).

RESULTS

The yellow compound obtained from the body fluid of the silkworm was dissolved in the $M/100$ phosphate buffer, pH 7.2 and this solution was analysed by means of paper chromatography, using *n*-butanol saturated with water. Two yellow spots were revealed on the chromatogram and their R_f values were 0.24 and 0.38 which agreed with those of authentic specimen of glyoxylic acid hydrazone. The appearance of two spots on the chromatogram of glyoxylic acid 2,4-dinitrophenylhydrazone is, as pointed out in the previous paper (*14*), due to the isomerisation of the molecule of the phenylhydrazone itself, which has a double bond between C and N, and which therefore may undergo a *cis-trans* isomerisation.

The yellow compound located at the position of R_f 0.24 on the chromatogram was eluted with $M/100$ phosphate buffer, pH 7.2 (A) or 1.25 N

sodium hydroxide (B), and the absorption spectra of the yellow or the red solution obtained were determined with Beckmann Spectrophotometer in the visible region of the spectrum (Fig. 1). The maximum absorption peak lay at 366–369 mμ in the case of (A) and 446–448 mμ in the case of (B) which agreed with those of the yellow compound located at the corresponding position of the chromatogram of authentic specimen of glyoxylic acid hydrazone. The absorption spectra of the yellow compound located at the position of R_f 0.38 on the chromatogram agreed also with those of the yellow compound located at the corresponding position of the chromatogram of authentic specimen of glyoxylic acid hydrazone.

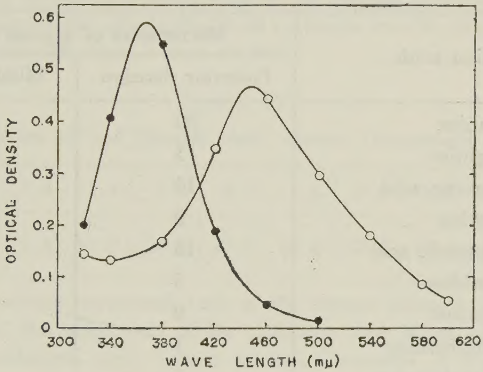


FIG. 1. Absorption spectra of 2,4-dinitrophenylhydrazone of glyoxylic acid. —●—, dissolved in $M/100$ phosphate buffer (pH 7.2), —○—, dissolved in 1.25 N NaOH.

TABLE I

Identification of Glyoxylic Acid

2,4-Dinitro-phenylhydrazones	*μg./ml.*	Optical densities at maximum absorption peak	Ratio of optical densities		
			400 : 440	400 : 520	420 : 520
α-Ketoglutaric acid	5–14	.205–.725	0.88–0.97	1.57–1.69	1.84–1.86
Oxalacetic acid	5–14	.203–.624	0.60–0.76	0.86–1.17	1.20–1.41
Glyoxylic acid	5–14	.070–.331	0.42–0.56	0.85–1.16	1.41–1.62
Pyruvic acid	5–14	.371–.846	0.54–0.63	0.83–0.97	1.26–1.34
Experimental		.247	0.56	1.10	1.50

In Table I are presented representative ratios of the absorption spectra of several known keto acids and that of the experimental. The ratios of the unknown were nearest to values obtained with glyoxylic acid hydrazone.

The yellow compound obtained from the body fluid

Found: N 21.98 per cent
Calcd. for $C_8H_6O_6N_4$: N 22.05 per cent

These results seem to suggest that the yellow compound obtained from the body fluid of the silkworm is 2,4-dinitrophenylhydrazone of glyoxylic acid.

A summary on the transaminase activities involving glyoxylic acid in the posterior division and in the middle division of the silkgland-cell is presented in Table II which shows the approximate amount of transamination as expressed in micromoles of glycine formed per 100 mg. of the fresh tissues. In the posterior division of the silkgland, it is especially worthy of note that glyoxylic acid—alanine transaminase is remarkably active, and glyoxylic acid—glutamic acid or glyoxylic acid—aspartic acid transaminases come next. On the contrary, in the middle division of the silkgland, the activities of the transaminase involving glyoxylic acid are very weak. Recently Nakada *et al.* (*15*) pointed out a phenomenon that glycine was also formed non-enzymatically from glyoxylic acid in the presence of amino acids. However, in such case that the enzyme solution was removed from the reaction mixture mentioned in the experimental section, the amount of glycine formed was always less than 1 μM, as shown in Table III, and was within the range of the measuring error. optimum pH of the transaminase reaction involving glyoxylic acid in the posterior division of the silkgland

TABLE II

Transaminase Activity of the Silkgland

Amino acids	Micromoles of glycine formed	
	Posterior division	Middle division
L-Alanine	30	1
L-Arginine	5	1
L-Aspartic acid	10	0
L-Cystine	9	1
L-Glutamic acid	13	0
L-Histidine	5	0
L-Leucine	0	0
DL-Methionine	1	1
L-Phenylalanine	0	0
DL-Serine	0	1
DL-Threonine	0	1
L-Tryptophan	3	0
L-Tyrosine	3	0
DL-Valine	7	0

The reaction mixture contained 1 ml. of enzyme solution prepared respectively from the posterior division and the middle division of the silkgland, 100 μM glyoxylate and 50 μM amino acids. Total volume 2.5 ml. Incubation time 1 hour at 38°.

TABLE III

Enzymatic or Non-Enzymatic Conversion of Glyoxylic Acid to Glycine in the Presence of Amino Acid

Amino acid added	Amounts of glycine formed (μM)	
	In the reaction added the enzyme solution*	in the reaction without added the enzyme solution
Alanine	40	0.4
Glutamic acid	15	0.3
Aspartic acid	9	0.3

* The reaction mixture contained 1 ml. of the enzyme solution prepared from the posterior division of the silkglands, 50 μM L-alanine, and 50 μM glyoxylate. Total volume, 3 ml. Incubation time, 1 hour at 38°.

TABLE IV

Relation between pH and Glyoxylic Acid—Alanine Transaminase Activity

pH	7.0	7.4	7.6	8.2	8.6	9.0	9.4	9.6	9.9	10.5
Amounts of glycine formed	11.4	17.9	18.5	27.5	28.6	32.2	43.1	41.2	36.1	25.0

The reaction mixture contained 1 ml. of the enzyme solution prepared from the posterior division of the silkglands, 50 μM L-alanine and 50 μM glyoxylate. Total volume, 3 ml. Incubation time, 1 hour at 38°. Amounts of glycine formed are expressed in μM.

was, as shown in Table IV, 9.4, being remarkably different from the value (optimum pH 7) which has been obtained in the tissues of higher animals. The phenomenon that the optimum pH of the transaminase reaction involving glyoxylic acid in the silkworm is 9.4 and lies in alkali side, is recently recognized also by Koide *et al.* (*7*) in the alimentary canal.

DISCUSSION

In the previous paper (*14*), it was clarified by paper chromatography that there were glyoxylic, α-ketoglutaric, oxalacetic and acetoacetic acid in the silkworm and especially glyoxylic acid had the highest concentration among these keto-acids in the body fluid and the silkgland: 30–50 mg. per dl. in the body fluid and 1–4 mg. per one hundred worms in the silkgland of the 5th instar. In the present experiment, glyoxylic acid was really isolated as its 2,4-dinitrophenylhydrazone from the body fluid of the silkworm larvae. Therefore, there will be no doubt about the occurrence of glyoxylic acid in the silkworm body. Also it was elucidated in the present paper that glycine was synthesized from glyoxylic acid by the transaminase, especially glyoxylic acid—alanine, next glutamic acid and aspartic acid transaminase in the posterior division of the silkgland. These findings seem to suggest that glyoxylic acid is one of the main precursors of the glycine synthesis in the silkworm.

Our observation that the transaminase involving glyoxylic acid in the posterior division is more active than that in the middle division may be of interest in relation to the fact (*16*) that posterior division of the silkgland is an organ for synthesis of fibroin containing a large quantity of glycine (42.8 per cent.) and the middle division is an organ for synthesis of sericin containing a small quantity of glycine (8.6 per cent.).

SUMMARY

1) The present work was carried out to examine whether glyoxylic acid is one of the main precursors of glycine synthesis in the silkworm.

2) Glyoxylic acid was isolated as its 2,4-dinitrophenylhydrazone from the body fluid of the silkworm larvae.

3) Glycine was synthesized from glyoxylic acid in the presence of amino acids, such as alanine, glutamic acid and aspartic acid, in the posterior division of the silkglands.

4) The transaminase involving glyoxylic acid in the posterior division of the silkgland was more active than that in the middle division.

5) The optimum pH of the transaminase involving glyoxylic acid was 9.4 and lay in alkali side.

6) These facts seem to suggest that the conversion of glyoxylic acid to glycine in the silkworm larva is of special importance in the synthesis of the glycine of the silk.

REFERENCES

(*1*) Meister, A., Sober, H. A., Tice, S. V., and Fraser, P. E., *J. Biol. Chem.*, **197**, 319 (1952)

(*2*) Weinhouse, S., and Friedmann, B., *J. Biol. Chem.*, **191**, 707 (1951)

(*3*) Wilson, D. G., King, K. W., and Burris, R. H., *J. Biol. Chem.*, **208**, 863 (1954)

(*4*) Campbell, L. L. JR., *J. Bact.*, **71**, 81 (1956)

(*5*) Wright, B. E., *Arch. Biochem. Biophys.*, **31**, 332 (1951)

(*6*) Kirimura, J., unpublished

(*7*) Koide, F., Shishido, T., Nagayama, H., and Shimura, K., *J. Agr. Chem. Soc. Japan*, **30**, 238 (1956)

(*8*) Doebner, O., *Ann. Chem.*, **311**, 129 (1900)

(*9*) Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932)

(*10*) Duchateau, G., and Florkin, M., *Arch. Internat. Physiol.*, **63**, 35 (1955)

(*11*) Fukuda, T., Kirimura, J., Matuda, M., and Suzuki, T., *Nature*, **175**, 1041 (1955)

(*12*) Giri, K. V., and Rao, N. A. N., *Nature*, **169**, 923 (1952)

(*13*) Giri, K. V., Radhakrishnan, A. N., and Vaidyanthan, C. S., *Nature*, **170**, 1025 (1952)

(*14*) Fukuda, T., Hayashi, T., and Matuda, M., *J. Japanese Biochem. Soc.*, **27**, 147 (1955)

(*15*) Nakada, H. I., and Weinhouse, S., *J. Biol. Chem.*, **204**, 831 (1953)

(*16*) Fukuda, T., Kirimura, J., Matuda, M., and Suzuki, T., *J. Biochem.*, **42**, 341 (1955)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

DISTRIBUTION OF INOSITOL IN THE FRACTIONS OF LIPIDES FROM BCG

BY SHOSHICHI NOJIMA, EIKO KONDO AND DEN'ICHI MIZUNO

(From the Department of Biochemistry, National Institute of Health, Tokyo, Japan)

(Received for publication, January 14, 1958)

It was from the lipides of tubercle bacilli that inositol was found for the first time as a component of lipides in nature (*1*). Since then inositol phosphatides have been found in animal as well as in plant kingdoms, and the recent investigations of purification, characterization and metabolism of inositol phosphatides have been very extensive and remarkable (*2*, *3*). As for tubercle bacilli, however, the study of inositol lipides was entirely concentrated on the so-called phosphatide fraction. Thus Anderson, in 1930, showed inositol to be one constituent of phosphatides in tubercle bacilli (*1*) and gave evidence that it is in the bound form with polysaccharide (*4*, *5*). Recently, Lederer showed further evidence that Phosphatide of both *H37 Rv* (*streptomycin resistant*) and *BCG* consist mainly of phosphatidylmannoinositose (*6*, *7*). On the other hand, content and behaviour of inositol in other fractions of lipides from tubercle bacilli have scarcely been considered.

The present work was undertaken to determine the inositol content in various fractions of lipides from *BCG* by the microbiological assay using *Saccaromyces carlsbergensis*. Contrary to our expectation, a great amount of inositol was found in the fraction of Wax D as well as in Phosphatide fraction.

MATERIALS AND METHODS

Microorganisms—The stock strain of *BCG* and *Aoyama B*, a human virulent *Mycobacterium tuberculosis*, supplied from the Department of Tuberculosis, National Institute of Health, Tokyo, were used in this work.

Culture—(*i*) *BCG*: After surface culture was made in Sauton's medium for 4 weeks, pellicles were filtered off, washed with water and promptly subjected to lyophilization. (*ii*) *Aoyama B*: Eight weeks culture in Sauton's medium for tuberculin production was used after autoclaving at 120° for 20 minutes followed by drying at 60°.

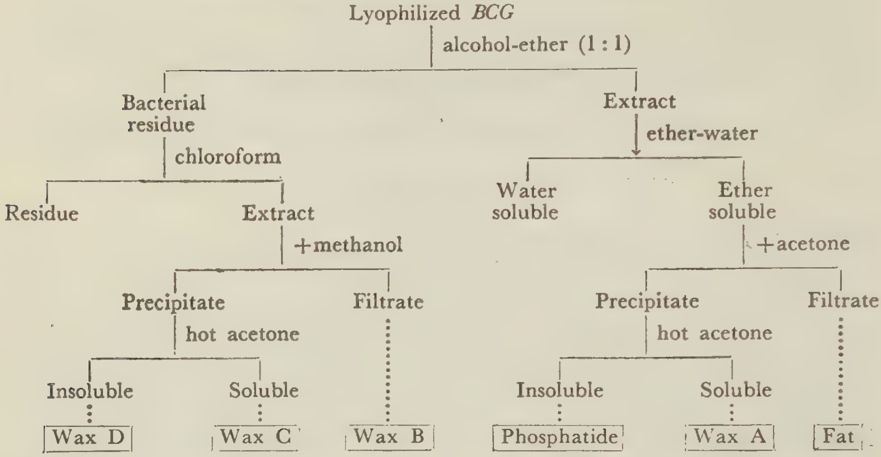
Other procedures for solvent extraction and fractionation of extracts from the bacterial cells were given in details by Asselineau (*8*) and Tsumita (*9*). Scheme 1 shows the extraction process.

Phosphorus was determined by Allen (*10*).

Nitrogen was determined by micro-Kjeldahl method and sometimes by Nesslerization using Conway's microdiffusion method.

SCHEME 1

Extraction and Fractionation of the Lipides from BCG



Estimation of Inositol—This was estimated by the microbiological assay with *Saccharomyces carlsbergensis* ATCC 9080 Strain No. 4228). The method was based on that of Atkin, Schulz, Williams and Frey (*11*) (see also (*12*)). Incubation was for 18 hours at 30° in 12×100 mm. tubes containing 2 ml. of medium: the tubes were incubated in a slope position to increase aeration. Growth was measured turbidimetrically with Coleman's spectrophotometer. Inositol could be assayed by this method from 0.1 μg to 1.25 μg with an accuracy of ±5 per cent.

Preparation of Sample for Inositol Assay—Samples (usually 10 mg) were hydrolyzed by refluxing for 40 hours in 6 *N* HCl. The hydrolysate was evaporated to dryness *in vacuo* and the residue was dissolved in water, filtered, diluted in an appropriate concentration, and neutralized with a small amount of $NaHCO_3$.

Paperchromatography for Carbohydrate—This was performed on filter paper (Whatman No. 1) at room temperature. The following solvent systems were used: pyridine-*n*-butanol-water (2 : 3 : 1.5) in case of ascending and ethylacetate-acetic acid-water (3 : 3 : 1) in case of descending. The spray reagents were aniline oxalate and ammoniacal silver nitrate (*13*).

RESULTS

Content of Phosphorus, Nitrogen and Inositol in Fractions of Lipides from BCG and Aoyama B—In the case of *BCG* it is shown from Table I that the content of inositol in both fractions of Phosphatide and Wax D was higher than in any other fractions. Phosphorus content in Phosphatide was almost the same amount as that in Phosphatide obtained by Asselineau (2.4 per cent), whereas that in Wax D of the present preparation was higher than his (0.20 per cent) (*8*). Similarly, in the case of *Aoyama B*, the results of which were shown as a control in Table II, inositol content in Phosphatide and Wax D is also higher than any other fractions. However, phosphorus (1.6 per cent) as well as inositol (0.60 per cent) content in the Phosphatide fraction of *Aoyama B* is lower than those of *BCG*.

TABLE I

Several Analytical Data of Various Fractions of Lipides from BCG

	Fat	Wax A	Phosphatide	Wax B	Wax C	Wax D
P (%)	0.0	0.0	2.73	—	0.0	0.80
N (%)	0.13	0.0	0.50	0.0	0.0	<0.2
Inositol (%)	0.05	—	2.79	0.42	0.0	3.18
Yield (%)	12.0	5.0	0.5	3.0	3.0	1.5

Yield is expressed in precentage as calculated from lyophilized cells.

TABLE II

Several Analytical Data of Various Fractions of Lipides from Aoyama B

	Fat	Wax A	Phosphatide	Wax C	Wax D
P (%)	0.0	1.6	0.09	0.0	0.76
N (%)	0.32	0.65	0.12	0.02	0.2
Inositol (%)	0.0	0.60	0.08	0.0	2.2
Yield (%)	11.2	0.8	2.0	0.7	3.9

TABLE III

Extraction Step of BCG by Alcohol-Ether and Chloroform

Solvent	No. of extraction	Days for extraction	Yields (g)
Alcohol-ether	1	3	2.60
	2	6	1.03
	3	8	0.40
	4	5	0.25
	5	6	0.13
Chloroform	6	3	0.67
	7	8	0.04

22 g. lyophilized *BCG* was subjected to extraction.

From the above experiments it may well be said that inositol lipides were involved in Wax D from *BCG* in amounts comparable to those in Phosphatide. However, since Phosphatides are the components of the alcohol-ether extracts which are least readily extractable by this mixture of solvents, extraction of another lot of *BCG* was carried out.

In this case, carefully lyophilized bacterial cells were extracted with alcohol-ether repeatedly and exhaustively until only a small amount of lipides (about 4 per cent of total alcohol-ether extract) was extracted by these solvents and finally placed in chloroform. The yield of Wax D on each step of extractions is shown in Table III. This preparation of Wax D showed a yield of 0.8 per cent of dried cells and the content of phosphorus and of inositol was 0.82 per cent and 2.91 per cent, respectively.

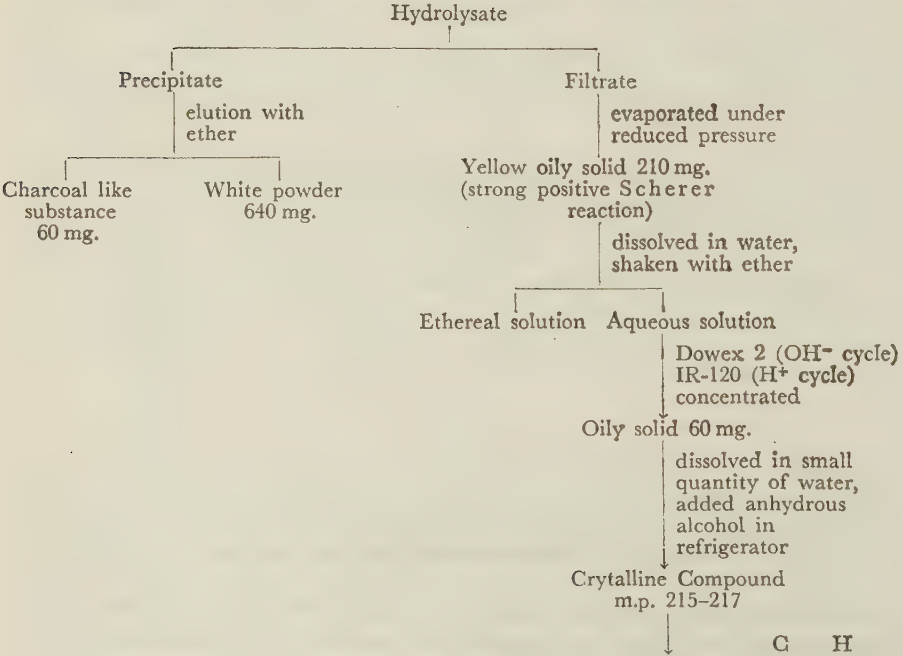
The above experiments show the following two facts: first it means that the reproducibility of extraction procedures was very high. Secondly, in the bacterial cells of lyophilized *BCG*, inositol lipides fraction which could not be extracted by alcohol-ether was present and could be extracted only by chloroform.

The Isolation of Inositol in Wax D—In order to confirm the analytical data shown above, isolation of inositol in Wax D was carried out. 1.0 g. of Wax D suspended in 60 ml. of 6 *N* HCl and refluxed for 37 hours in an oil bath. After cooling 25 mg of crystalline inositol was isolated according to the procedures shown in Scheme 2.

SCHEME 2

Hydrolysis of Wax D from BCG by HCl

1 g. of Wax D from BCG was refluxed with 60 ml. of *N* HCl for 37 hours.


Hydrolysate
Precipitate
elution with ether
Charcoal like substance 60 mg.
White powder 640 mg.
Filtrate
evaporated under reduced pressure
Yellow oily solid 210 mg. (strong positive Scherer reaction)
dissolved in water, shaken with ether
Ethereal solution
Aqueous solution
Dowex 2 (OH⁻ cycle)
IR-120 (H⁺ cycle)
concentrated
Oily solid 60 mg.
dissolved in small quantity of water, added anhydrous alcohol in refrigerator
Crytalline Compound
m.p. 215–217

	C	H
Calcd. for $C_6H_{12}O_6$:	40.0	6.67 (%)
Found:	39.49	6.70 (%)

500 mg. Wax D was dissolved in 50 ml. of ether and the ethereal solution was shaken with 100 ml. of water for 10 hours at room temperature. The resulting aqueous solution gave a negligible residue which did not show

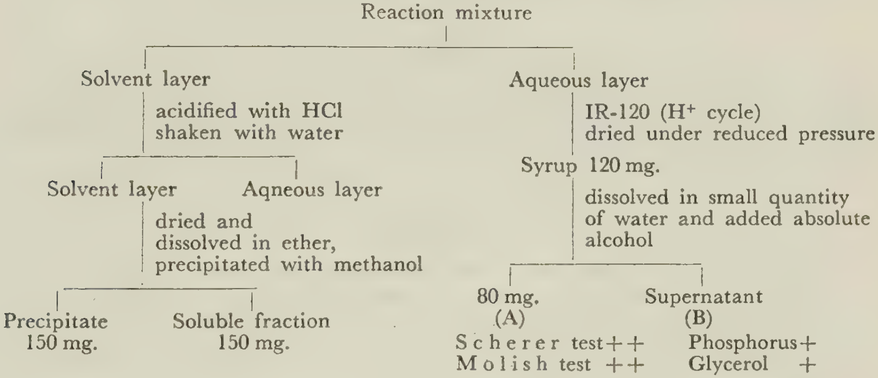
Scherer test. Thus, inositol was proved to be chemically combining with lipides in Wax D from *BCG*.

Examination of Other Components in Wax D—The results of alkaline hydrolysis of Wax D are shown in Scheme 3. 500 mg. Wax D was dissolved in 5 ml. of benzene and 5 ml. of 5 per cent of methanolic KOH was added. After refluxing for 4 hours on water bath, ether and water were added to give two layers, each of which was treated as shown in Scheme 3.

Scheme 3

Hydrolysis of Wax D from BCG by Alkali

0.5 g. of Wax D from *BCG* was dissolved in 5 ml. of benzene and this solution was refluxed for 4 hours, adding 5 ml. of 5 per cent of methanolic KOH.



10 mg. of (A) (Molish and Scherer positive) was dissolved in 5 ml. of 5 per cent H_2SO_4, heated for 2 hours in water bath, neutralized with $BaCO_3$ after cooling and the supernatant solution was isolated by centrifugation. The barium was removed with IR-120 (H^+ cycle) and the solution was concentrated to 1 ml. *in vacuo*, and paperchromatographed according to the methods described above. Mannose, inositol and a small amount of glucose were identified. Glycerol and phosphorus were shown qualitatively.

DISCUSSION

Anderson isolated inositol in purified Wax from human as well as from bovine type of tubercle bacilli in an amount of 0.05 per cent and 0.3 per cent as calculated from Wax, respectively (*14*, *15*). Asselineau described arabinose, galactose and mannose in water soluble fractions after the hydrolysis of Wax D of human types while he did not mention at all about inositol* (*8*, *9*). By means of microbiological assay which is the most reliable method for the determination of inositol, 3 per cent of inositol was found in Wax D of *BCG* and this fact was further confirmed by the isolation of an equal amount of inositol in a crystalline form. Whether inositol contained in Wax D is in the form of inositol phosphatide or in other forms is an interesting problem. In our further studies it has been shown by column chromatography on silicic acid that one of the main fractions of Wax D of *BCG* was inositol phosphatide. Details will be given in the near future.

---

* Asselineau later confirmed inositol and glycerol in the water soluble part after the hydrolysis of Wax D of several human strains of tubercle bacilli (personal communication). His preparation kindly supplied by himself (Canetti strain of human type) gave 0.7 per cent of inositol as determined by us.

In view of extraction technique for natural products it is of interest that there were two kinds of inositol lipides—one is extractable by alcohol-ether, while the other only by chloroform. Whether or not these two lipides are identical with each other remains to be ascertained.

SUMMARY

The content of inositol in all lipide fractions from *BCG* was determined by the microbiological assay. It was shown that 3 per cent of inositol was found in the Wax D fraction.

REFERENCES

(*1*) Anderson, R. J., *J. Am. Chem. Soc.*, **52**, 1607 (1930)
(*2*) Folch, J., and LeBaron, F. N., *Can. J. of Biochem. and Physiol.*, **34**, 305 (1956)
(*3*) Dawson, R. M. C., *Biological Review*, 32, 188 (1957)
(*4*) Anderson, R. J., Lothrop, W. C., and Creighton, M. M., *J. Biol. Chem.*, **125**, 299 (1938)
(*5*) de Sütö Nagy, G. I., and Anderson, R. J., *J. Biol. Chem.*, **171**, 749, 761 (1947)
(*6*) Vilka, E., and Lederer, E., *Bull. Soc. Chim. Biol.*, **38**, 111 (1956)
(*7*) Vilka, E., *Compt. Rend. Acad. Science*, **245**, 588 (1957)
(*8*) Asselineau, J., Thèse Doctorat, Paris 1950 p. 57 Ed., Arnette, 1951
(*9*) Tsumita, T., *Jap. J. Med. Sc. and Biol.*, **9**, 205 (1956)
(*10*) Allen, R. J. L., *Biochem. J.*, *34*, 858, (1940)
(*11*) Atkin, L., Schulz, A. S., Williams, W. L., and Frey, C. N., *Industr. Engng. Chem.*, (Anal.), **15**, 141 (1943)
(*12*) Nojima S., *J. of Japan. Chem.*, (1958) *in press*
(*13*) Block, R. J., Durrum, E. L., and Zweig, G., A Manual of Paper Chromatography and Electrophoresis, Academic Press, 132, 133 (1955)
(*14*) Robert, G., and Anderson, R. J., *J. Biol. Chem.*, **90**, 33 (1931)
(*15*) Cason, J., and Anderson, R. J., *J. Biol. Chem.*, **126**, 527 (1938)
(*16*) Aebi, A., Asselineau, J., and Lederer, E., *Bull. Soc. Chim. Biol.*, **35**, 661 (1953)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

STUDIES ON THE BIOSYNTHESIS OF SILK FIBROIN

I. INCORPORATION OF GLYCINE-1-C^{14} INTO FIBROIN IN VIVO

BY KENSUKE SHIMURA, HIRONOBU FUKAI, SEIICHI SUTO
AND REIKO HOSHI

*(From the Laboratory of Biochmistry, Department of Agriculture,
Tohoku University, Sendai)*

(Received for publication, February 3 1958)

Silk fibroin has been investigated in detail on the chemical and physical side, because of its highly characteristic molecular structure and its very simple amino acid composition. For all many previous studies, however, we are unfortunately able to say very little about the mechanism of biological synthesis of silk fibroin. On the early stage of our investigation on the fibroin formation we demonstrated an *in vivo* formation of silk protein by incubating a silk-gland in a synthetic medium (*1*). Sissakian and Kuvayeva (*2*) observed also an increased synthesis of protein in the culture medium containing amino acids and body fluid of pupa of silkworm. Zamecnik and his coworkers (*3*) showed incorporation of radioactive glycine and alanine injected into body cavity of silkworms into the protein of the cocoons. More recently incorporation of radioactive phenylalanine into silk protein was reported by Fukuda (*4*) and Bricteux-Gregoire *et al.* (*5*).

In the present paper incorporations of glycine-1-C^{14} into the silk-gland and silk fibroin were studied in greater details.

MATERIALS AND METHODS

Silkworms (*Bombyx mori*, L: a hybrid "Si 122 X Nichi 122") at the third to fifth day of the fifth instar were used for the injection of radioactive amino acid.

Radioactive Amino Acid—Glycine-1-C^{14} (0.1 mc per 2.12 mg.) was obtained from the Radiochemical Centre, Amersham, England.

Isotope Analysis—Radioactivity measurements were made on a Geiger-Müller counter with a thin mica window (1.43 mg. per cm^2). Suitable corrections were applied for background and self-absorption within the sample. Sufficient counts were taken so that the standard deviations were less than 5 per cent. All protein samples were finely powdered and dried over concentrated sulfuric acid.

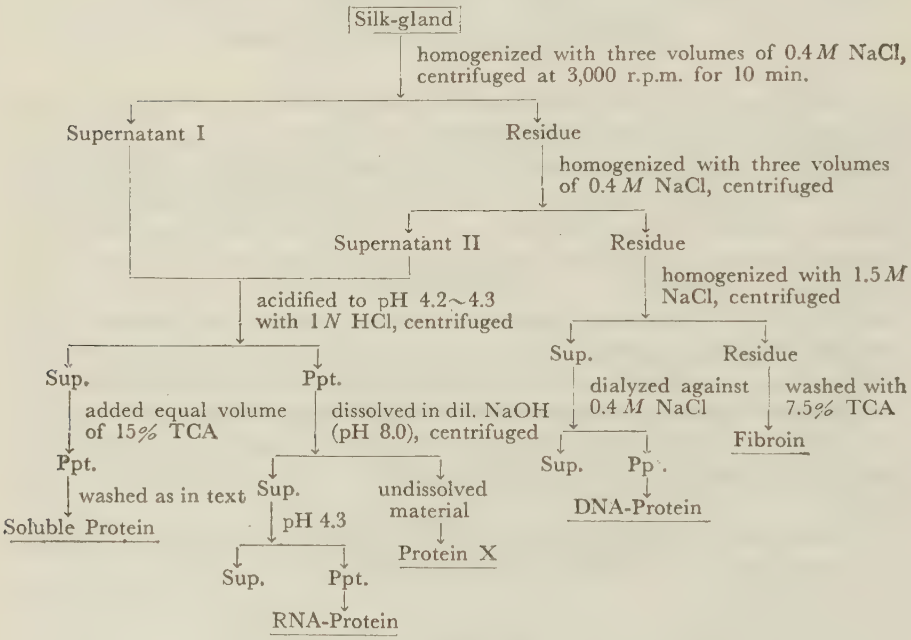
Injection and Preparation of Tissue Protein—Radioactive amino acid dissolved in 0.05 ml. of 0.9 per cent saline was injected into the body cavity of silkworm. At different time intervals after the injection a bowel leg. of the silkworm was cut and the effusing blood was collected quickly into an ice-cold test tube, and then the silkworm was dissected to get the silk-glands and other tissues. Each tissue was homogenized in a glass homogenizer, added equal volume of 15 per cent trichloroacetic acid, and allowed to stand overnight. The precipitates were washed five times with cold 7 per cent trichloroacetic acid, two times with ethanol-ether (1 : 1) and finally with ether.

Fractionation of Protein in the Posterior Silk-Gland—Two methods were carried out.

a) *Griffin's Method*: Fibroin, ribonucleoprotein (RNAP), desoxyribonucleoprotein (DNAP) and soluble protein in the posterior silk-gland were separated by a modified method of Griffin (*6*). The details of the fractination procedure are given in Scheme 1. The each protein fraction was precipitated with trichloroacetic acid and washed throughly as described above.

SCHEME 1

Scheme for Fractionation of the Proteins of Posterior Silk-Gland


Silk-gland
homogenized with three volumes of 0.4 M NaCl, centrifuged at 3,000 r.p.m. for 10 min.
Supernatant I
Residue
homogenized with three volumes of 0.4 M NaCl, centrifuged
Supernatant II
Residue
homogenized with 1.5 M NaCl, centrifuged
acidified to pH 4.2∼4.3 with 1 N HCl, centrifuged
Sup.
Ppt.
Sup.
Residue
added equal volume of 15% TCA
dissolved in dil. NaOH (pH 8.0), centrifuged
dialyzed against 0.4 M NaCl
washed with 7.5% TCA
Ppt.
Fibroin
Sup.
Ppt.
washed as in text
Sup.
undissolved material
DNA-Protein
Soluble Protein
pH 4.3
Protein X
Sup.
Ppt.
RNA-Protein

b) *Trichloroacetic Acid Method* (*TCA Method*): The excised posterior silk-glands were steeped in 5 per cent trichloroacetic acid solution at room temperature for 5 hours. The coagulated tissue proteins were separated with the aid of small pin-cettes from the fibroin, which is easily distinguished from the tissue protein by its jelly like look.

Separation of Cytoplasmic Granules from the Posterior Silk-Gland—For the separation of particulate components the sucrose fractionation method of Schneider (*7*) was employed. The posterior silk-glands were homogenized with ten volumes of 0.25 *M* sucrose solution at 0° for one minute. After removal of the insoluble protein and nuclei by sedimentation at 600×*g* for 10 minutes, large granular fraction was sedimentated at 8,500×*g* for 10 minutes and washed once with 0.25 *M* sucrose. From the combined supernatant small granular fraction was obtained by centrifugation at 18,000×*g* for 60 minutes or 105,000×*g* for 60 minutes. The both granules were washed two times with seventy per cent ethanol, once with 94 per cent ethanol, absolute ethanol and ether.

The supernatant from the small granule preparation contained the soluble non-particulate components of the cell. These proteins were precipitated with 10 per cent trichloroacetic acid and designated as soluble protein.

Ion Exchange Chromatography—For the assay of location of radioactivity in the radioactive fibroin the excellent method of Hirs *et al.* (*8*) for amino acid fractionation with Dowex-50 and Amberlite IR-4B was employed. The columns used in the present work were 0.9 cm. in diameter and 120 cm., 30 cm. and 15 cm. in length, repectively. Ammonium formate and ammonium acetate buffers were used. The collection of effluents was carried out by an automatic fraction collector. The course of the elution was followed by colorimetrical measurement of ninhydrin reaction and by measurement of radioactivity.

RESULTS

Incorporation of C^{14}-Glycine into Various Tissue Proteins—Glycine-1-C^{14} (1.4×10^5 c.p.m.) dissolved in 0.9 per cent saline was injected into silkworm. At the time of 3 minutes to 48 hours after the injection, the insects were dissected and their posterior silk-glands, middle silk-glands, intestinal tissues, dermal tissues and blood were collected and homogenized with an equal volume of 0.9 per cent saline as rapidly as possible. The proteins of each tissue were precipitated with trichloroacetic acid and washed as described above, except that the blood protein was precipitated in 70 per cent ethanol. The radioactivities (c.p.m. per mg.) of each protein fraction are set forth in Fig. 1. It is a most remarkable fact that a great part of the radioactive glycine was incorporated into the posterior silk-glands during the first four hours, but only at small extent into other proteins. However, the radioactivity of the posterior glands began to decrease gradually after about 10 hours. This is in contrast to the radioactivity change of the middle glands, of which radioactivity increased gradually and was about ten times of that of the posterior gland at the end of 48 hours. These facts present a clear evidence that silk fibroin is synthesized in the posterior gland and transfered

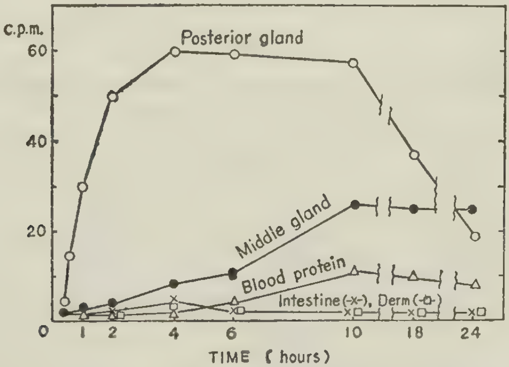


FIG. 1. Incorporation of glycine-1-C^{14} into the proteins of the various tissues of silkworm. The specific radioactivities are expressed as c.p.m. per mg. of protein.

mostly into the middle gland within 48 hours.

It is also clear that the radioactivity of blood protein is much less than that of the posterior gland at each time. This result may be interpreted as indicating that the blood protein is not a precursor protein for fibroin synthesis.

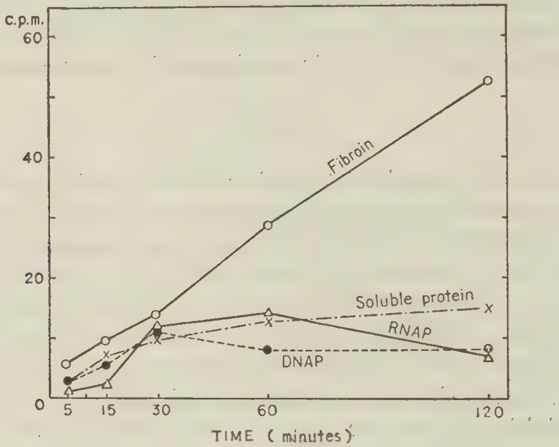


FIG. 2. Incorporation of glycine-1-C^{14} into the proteins of posterior silk-gland.

TABLE I

Specific Radioactivity of Fibroin and Tissue Protein Prepared by TCA and Griffin's Method

Time after injection	Fibroin				Tissue	
	Exp. I*		Exp. II*		Exp. I	Exp. II
	TCA	Griffin	TCA	Griffin	TCA	TCA
	(*c.p.m./mg.*)	(*c.p.m./mg.*)	(*c.p.m./mg.*)	(*c.p.m./mg.*)	(*c.p.m./mg.*)	(*c.p.m./mg.*)
10 mins.	0.2	33.3			30.4	
20 mins.	0.4	36.7			40.3	
30 mins.			2.8	40.5		34.4
90 mins.			32.8	50.0		21.4
4 hrs.			129.7	31.2		7.5
18 hrs.			39.1	14.4		1.2

* Injected 30,000 c.p.m. of glycine-1-C^{14} per silkworm.

Incorporation of C^{14}-Amino Acid into Various Protein Fractions of the Posterior Silk-Gland—As the rapid incorporation of glycine-1-C^{14} into the posterior gland was confirmed, it was undertaken, furthermore, to isolate fibroin, ribonucleoprotein, desoxyribonucleoprotein and soluble protein from the posterior gland. The change in the specific radioactivity of these proteins after the injection of glycine-1-C^{14} (1.4×10^5 c.p.m.) is shown in Fig. 2 (Griffin's method). The specific radioactivity of fibroin seems always to be higher than these of other fractions. That is, under these conditions, we could not show evidently whether there is a stage in which the radioactivity of RNAP or DNAP is higher than that of fibroin.

In Table I a typical result with TCA method is presented. There may be seen a distinct difference between the radioactivity change of fibroin prepared by Griffin's and TCA method. Labeling of the fibroin prepared by the TCA method reveals clearly a lag phase during first 30 minutes after injection. It is of interest that the tissue protein is more radioactive than the fibroin in this phase.

Incorporation of C^{14}-Amino acid into the Large and Small Granules of Posterior Silk-Gland—The results are shown in Fig. 3. The specific radioactivities of the large and small granules are maximum at around 20 minutes after injection, and much higher than that of fibroin. It is noteworthy that the mitochondrial protein has higher activity than the microsomal protein.

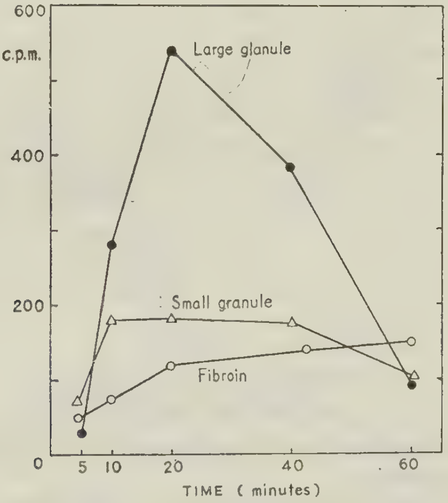


FIG. 3. Incorporation of glycine-1-C^{14} into the granules of posterior silk-gland.

Location of Radioactivity in Amino Acids of Fibroin—Dowex-50 column chromatography was used to localize the radioactivity among the amino acids in the hydrolyzate of radioactive fibroin, which was isolated from posterior silk-gland at four hours after injection of glycine-1-C^{14}. The chromatographic results are shown in Fig. 4, in which a shaded portion represents the radioactivity of effluent. The chromatogram shows clearly that about 95 per cent of the radioactivity recovered is located in glycine and small amount of activity is in serine (3 per cent) and alanine (1 per cent). This result indicates that the most part of radioactivity of fibroin is due to its glycine residues.

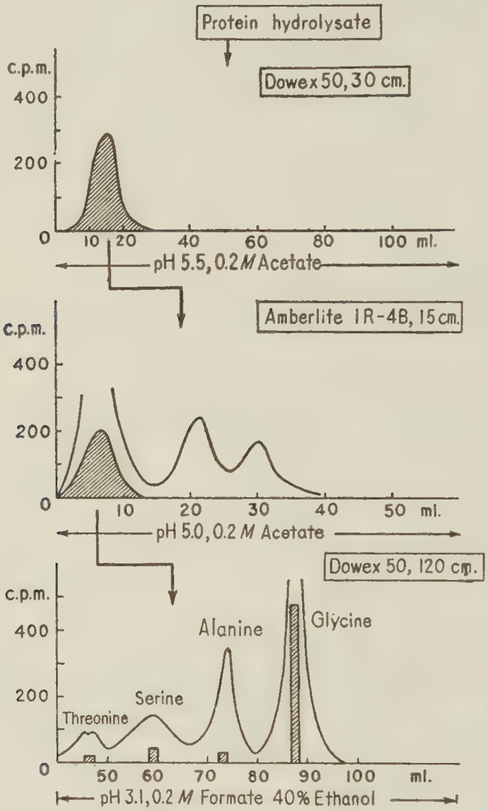


FIG. 4. Ion exchange chromatograms of the hydrolysate of radioactive fibroin.

The shaded portions represent the radioactivities of effluents and the solid lines are ninhydrin determinations, 1 ml. of the hydrolysate of 10 mg. of radioactive fibroin (60 c.p.m. per mg.) was placed on the column.

DISCUSSION

A great part of glycine-1-C^{14} injected into body cavity of silkworm was shown to be incorporated into the posterior silk-gland. In the experiment described in Table I, the change of specific radioactivity of fibroin prepared by TCA method shows a distinct lag phase during first 30 minutes after injection as compared with the fibroin prepared by Griffin's method. There are two possible explanations for the appearance of this lag phase. First, we can expect a formation of precursor protein in the gland tissue, because the radioactivity of free glycine in the blood decreased rapidly during first 30 minutes after injection and the tissue protein, on the other hand, showed considerably high radioactivity even at 10 minutes after injection. This fact may suggest that the radioactive amino acid is incorporated into the precusor protein in the gland tissue and then the precursor protein converts into fibroin. Another possible explanation is that the radioactive protein in the gland tissue is a fibroin itself which is synthesized newly, and the lag phase indicates a time for the secretion of synthesized fibroin. The characterization of this radioactive protein in the gland tissue is now under investigation.

The fact that the fibroin preparation obtained by the Griffin's method did not reveal a lag phase will be interpreted as a contamination of this tissue protein, which is insoluble in 0.4 *M* and 1.0 *M* NaCl, and has similar properties to fibroin. The TCA method, therefore, seems to be better than the Griffin's method to get a pure fibroin from the posterior silk-gland.

The result in Fig. 3, shows a more active uptake of radioactive amino acid into the protein of the large granule fraction, which is about twofold as radioactive as the small granule and threefold as fibroin. These facts are not consistent with the results of other workers (*9*, *10*, *11*). They demonstrated that the microsomal fraction of rat or mice liver incorporates amino acids at much greater rate than does any other fractions.

Now there arises a question whether the large and small granules which we isolated are actually correspond to mitochondrial and microsomal fractions in a usual sense or not. Some analytical data concerning the properties of both particles are shown in Table II. The small granule is more rich in RNA and has less succinoxidase activity than the large granule. These results support that the large and small granules are correspond to mitochondria and microsomes of mammalian liver, respectively. Therefore, the idea that microsome is the actual loci for synthesis of protein but that simultaneous oxidative phosphorylation by mitochondria also necessary

TABLE II

Amounts of Total Nitrogen and Ribonucleic Acid Phosphorus (RNA-P) in the Cell Particles of Postirior Silk-Gland

Particles	Total N	PNA-P	RNA3)	$\frac{\text{Total N}}{\text{RNA-P}}$	Succinoxidase activity4)
	(*μg./mg.*)	(*μg./mg.*)	(%)		(Q_{O_2}/*mg.* N)
Large granule1)	100.4	10.2	10.8	9.8	462
Small granule2)	105.4	19.8	21.0	5.3	73

1) Centrifuged at 8,500×*g* for 10 minutes.

2) Centrifuged at 105,000×*g* for 60 minutes.

3) Calculated assuming RNA contains 9.4 per cent phosphorus.

4) Determined by the method of Schneider *et al.* (*J. Biol. Chem.*, **149**, 217 (1953)).

seems to be inappropriate to apply to the cellular system of posterior silk-gland.

SUMMARY

1. A large part of glycine-1-C^{14} injected into the body cavity of silkworm was incorporated into the posterior silk-gland.

2. Two methods (Griffin's and TCA method) were attempted to fractionate the proteins of the posterior silk-gland. The change of specific radioactivity of fibroin prepared by TCA method showed a lag phase during first 30 minutes after the injection.

3. The large granule of the cells of the posterior silk-gland incorporated glycine-1-C^{14} more than the small granule.

4. Location of radioactivity in amino acids of fibroin was determined by a method of Dowex-50 column chromatography. About 95 per cent of the total radioactivity recovered was shown to be located in glycine residue.

REFERENCES

(*1*) Shimura, K., Koide, F., Itabashi, H., and Fukai, H., *J. Biochem.*, **42**, 285 (1955)

(*2*) Sissakian, N. M., and Kuvayeva, E. B., *Biokhimia*, **18**, 354 (1953) : Intern. Symposium on Enzyme Chem. at Tokyo, p. 314 (1957)

(*3*) Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L., and Williams, C. M., *Science*, **109**, 137 (1949)

(*4*) Fukuda, T., *J. Biochem.*, 43, 137 (1956)

(*5*) Bricteux-Gregoire, S., Verly, W. G., and Florkin, M., *Nature*, **177**, 1237 (1956)

(*6*) Griffin, A. C., Nye, W. N., Nod, L., and Luck, J. M., *J. Biol. Chem.*, **176**, 1225 (1948)

(*7*) Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950) ; **172**, 619 (1948)

(*8*) Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **195**, 669 (1952)

(*9*) Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol Chem.*; **187**, 839 (1950)

(*10*) Keller, E. B., and Zamecnik, P. C., *Federation Proc.*, **10**, 206 (1951)

(*11*) Lee, N. D., Anderson, J. T., Miller, R., and Williams, R. H., *J. Biol. Che* **192**, 733 (1951) ; **200**, 451 (1953)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

INCORPORATION OF RADIOACTIVE PHOSPHATE INTO PROTEIN-BOUND PHOSPHORUS FRACTIONS OF BRAIN SLICES IN REFERENCE TO ITS RELATION TO THE METABOLIC ACTIVITY

By YASUZO TSUKADA, GENKICHIRO TAKAGAKI
AND SHUSUKE HIRANO

(*From the Department of Physiology, School of Medicine,
Keio University, Tokyo*)

(Received for publication, February 24, 1958)

Previously the effect of changing the environment on the metabolic patterns in the cerebral cortex slices has been reported (*1–4*). As well known, an increase of potassium ion concentration, or an addition of L-glutamate, causes an increase in the oxygen consumption and aerobic glycolysis of brain slices (*5–7*), while it decreases their concentration of phosphocreatine (*2*, *8*). Therefore, it may be thought that the uncoupling of oxidative phosphorylation would be brought about thereby similary as by the addition of 2,4-dinitrophenol. On the other hand, it has already been shown that the incorporation of inorganic P^{32} into the pentosenucleic acid, phospholipid and other protein-bound phosphorus compounds of respiring brain slices is dependent upon oxidative phosphorylation mechanism (*9–12*). These considerations prompted a detailed experiment using P^{32} to disclose further the relation between the energy yielding metabolism and the phosphorylation of brain slices.

In the experiments, the incorporation of inorganic P^{32} into "phosphoprotein" of brain slices was indicated to be increased, under the conditions where enhancement of metabolism was observed, by increasing the concentration of potassium ions. Accordingly the evidence was presented that the increased metabolism of brain slices by the addition of potassium ions is coupled with the incorporation of P^{32}. The effects of other substances on P^{32} incorporation were also described concerning with their relation to the changes of metabolism. The special effect of L-glutamate on brain slices was also discussed.

A preliminary report of these experiments has already appeared (*13*).

METHODS

Preparation and Incubation of Brain Slices—Guinea pig brain slices were prepared on the cooled stage with a safety razor blade from the cerebral hemispheres and stored in ice cold Krebs-Ringer solution. Slices of 0.3–0.4 mm. thick weighing 300–400 mg. in wet weight were transferred to Warburg flasks containing 10 ml. Krebs-Ringer solution buffered with glycylglycine (pH 7.0). Into each flasks 50 μc of radioactive phosphate ($H_3P^{32}O_4$) and 0.011 M glucose were added. The flasks were aerated with pure oxygen and then incubated at 37° for 90 minutes with shaking.

The basic saline medium used contained 0.127 M NaCl, 0.0051 M KCl, 0.00273 M $CaCl_2$, 0.00134 M KH_2PO_4, 0.00134 M $MgSO_4$, 0.05 M glycylglycine brought to pH 7.0 with N NaOH, and 0.011 M glucose (*14*).

The high potassium medium was made by the addition of KCl to Krebs-Ringer solution without the correction of osmotic pressure. When the sodium-free medium was prepared, sodium ions were replaced by choline to maintain the isotonicity of the medium, and the glycylglycine was neutralized with N KOH. In preparing a potassium-free medium, KH_2PO_4 was replaced by NaH_2PO_4. The additional ammonium ions were added as NH_4Cl. For calcium-free medium, $CaCl_2$ was omitted from the basic Ringer solution. The tricarboxylic acid cycle intermediates were added as the sodium salts. When oxygen uptake, glucose utilization, lactic acid formation and activity of transamination of brain slices were to be estimated without determining the incorporation of P^{32}, phosphate was used instead of glycylglycine as a buffer, and the concentration of glucose in the medium was reduced to 3.3×10^{-3} M.

Determination of the Specific Activities—The fractionation of the phosphoric esters of brain slices for the determination of the specific activities was performed principally as described by Friedkin and Lehninger (*15*).

After the incubation period, the slices were transferred rapidly to a Potter-Elvehjem type homogenizer containing 6 ml. of ice cold 10 per cent (W/V) trichloroacetic acid (TCA) and then homogenized for 30 seconds. The suspension was centrifuged carefully, and the supernatant was treated twice with magnesia mixture to remove inorganic phosphate and then ashed with perchloric acid. This acid soluble fraction was taken for counts and for phosphate determinations.

The TCA precipitate was washed twice with 10 per cent TCA. The lipid in the washed precipitate was removed by two extractions with 5 ml. of 95 per cent (V/V) ethanol and two extractions with 5 ml. of ethanol-ether (3 : 1) for 5 minutes at 60°. The nucleic acids were then removed from the protein residue by three extractions with 5 ml. of 5 per cent (W/V) TCA for 15 minutes at 95°, and finally washed with 5 per cent cold TCA. The residue was taken up in 3.0 ml. of N NaOH and kept at 37° for 15 hours. Then, 0.6 ml. of 6 N HCl and 3 ml. of 10 per cent TCA were added and centrifuged. The inorganic phosphorus of the acid-soluble portion of the hydrolyzate is derived from the hydrolysis of "phosphoprotein". The organic P of the hydrolyzate in the acid-soluble fraction, after two further magnesia treatments, forms the residual organic P.

In most experiments, the so-called protein-bound P contains both the phosphoprotein and the residual organic P fractions. These organic P fractions were ashed completely with 1 ml. of 60 per cent perchloric acid, and then phosphorus was estimated by the method of Martin and Doty (*16*). Both the concentration and the radioactivity of the inorganic P were determined on the same sample. The radioactivity in the sample was counted by a GM counter. The counting rate was corrected for background and decay, and the specific activities were expressed as the number of counts per μg. of phosphorus per minute.

Other Analytical Methods—Oxygen uptake was measured by the usual manometric techniques at 37° in pure oxygen. Glucose utilization was estimated using the anthrone reagent as described by Koehler (*17*). Lactic acid formation in the medium was determined chemically by the method of Barker and Summerson (*18*). Aspartic acid content in respiring slices was measured paperchromatographically according to the method described by Awapara *et al.* (*19*). The paper used was of No. 51 (4×40 cm.)

of Toyo-Roshi Co., Tokyo. Ninhydrin reagent was prepared by the method of Yemm and Cocking (*20*). Reagent blanks against 50 per cent ethanol were usually about 0.060 optical density units at 570 mμ, and paper blanks were about the same degree. The optical density of 0.2 μM of aspartic acid was about 0.320.

RESULTS

P^{32} Incorporation and Potassium Effect on Brain Slices—Table I shows the changes in the specific activities of acid-soluble P and protein-bound P of guinea pig brain slices under various conditions with or without high concentration of KCl. The results were expressed as a percentage of the control metabolizing in the basic glucose-containing medium.

TABLE I

Effect of the Addition of KCl on the P^{32} Incorporation into the Acid-Soluble P and Protein-Bound P of Guinea Pig Brain Slices under the Various Conditions

Condition	Addition of KCl (10^{-1} *M*)	Acid-soluble P (%)	Protein-bound P (%)
Glucose	+	136±32*(7)	182 ±28*(7)
Glucose and malonate	+	17.0	23.7±11*(3)
ditto	−	24.5	30.7±14*(3)
Glucose and DNP	+	13.0	26.7
ditto	−	12.9	17.7
Pyruvate	+	63.2	54.4
ditto	−	52.6	60.2
No substrate	−	32.6	41.5

The incorporations in four or five different conditions, including every time a normal control one, were examined at the same time and the changes in the specific activities were calculatard relatively to that control.

* Mean ± standard deviation; the value without standard devitation, the mean of two experiments; figure in parentheses, number of experiments.

By the addition of potassium ions (10^{-1} *M*) to the usual medium, the incorporation of P^{32} into protein-bound P of the cerebral cortex slices was apparently increased, and also that into acid-soluble P was increased slightly.

Although sodium malonate (10^{-2} *M*), as well known, does not inhibit the respiration of brain slices (*21*), the incorporation of P^{32} into phosphorus compounds of them was markedly depressed by the addition of malonate, either in the presence or in the absence of high concentration of potassium ions.

2,4-Dinitrophenol (5×10^{-5} *M*) also augments both the oxygen uptake and lactic acid formation as well as the presence of high concentration of potassium ions does. On the contrary the incorporation of P^{32} was depressed strongly by 2,4-dinitrophenol.

In the case where pyruvate was used as a substrate instead of glucose, the incorporation of P^{32} into acid-soluble P and protein-bound P was not increased even by the addition of potassium ions.

Table II shows the effect of potassium ions on the specific activities of protein-bound P of guinea pig liver slices metabolizing glucose. It was indicated that an increase in the concentration of potassium ions has no effect on the incorporation of P^{32} by liver slices. The increase of incorporation of P^{32} into protein-bound P coupled with metabolism in the presence of high concentration of potassium ions is thought to be peculiar to the brain slices respiring in glucose-containing medium.

TABLE II

Specific Activity of the Protein-Bound P of Guinea Pig Liver Slices Incubated in Potassium-Rich Krebs-Ringer Glycylglycine Buffer Containing Glucose and P^{32}

Condition	Specific activity (*c.p.m./μg* P) of protein-bound P		
	Expt. 1	Expt. 2	Expt. 3
Normal medium	209 (1510)	660	580
K^+ rich medium	256 (1750)	670	390

Slices incubated for 90 minutes at 37° in Krebs-Ringer solution. Figure in parentheses indicates the specific activity of the acid-soluble P.

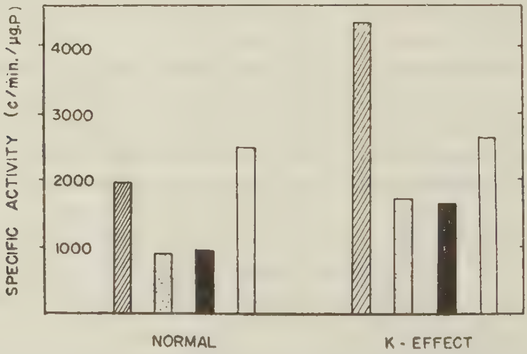


FIG. 1. Effect of the addition of K^+ on the specific activity of phosphoprotein P (PP), residual organic P (ROP) and residue P (RP) of guinea pig brain slices.

The height of each column shows the mean value of the specific activities (c.p.m./μg. P) of PP (▨), ROP (░), RP (■) and acid-sol. P (□), obtained in three independent experiments. Slices incubated for 90 minutes at 37° in medium containing glucose and P^{32} (60 μc). In K-effect, KCl (10^{-1} *M*) was added to the basic medium.

Fig. 1 presents the results of an experiment in which the specific activities of phosphoprotein, residual organic P and insoluble residue were determined respectively at the same time according to the method of Findlay *et al.* (*9*). Owing to potassium effect the specific activity of the phosphoprotein fraction was increased to the highest degree among these fractions.

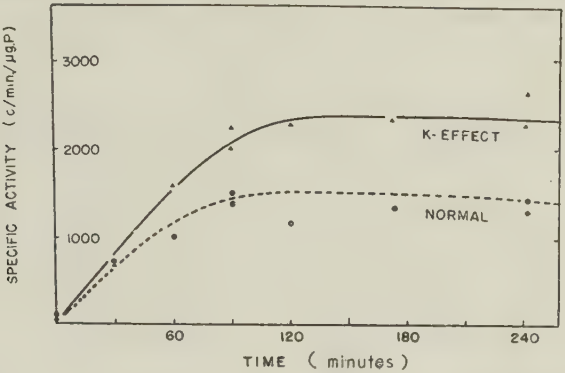


FIG. 2. Time course of the incorporation of P^{32} into protein-bound P of brain slices respiring in the medium containing P^{32} in the presence (▲—▲) or in the absence (●···●) of KCl (10^{-1} *M*). Point (▲, ●) in the figure represents each estimation.

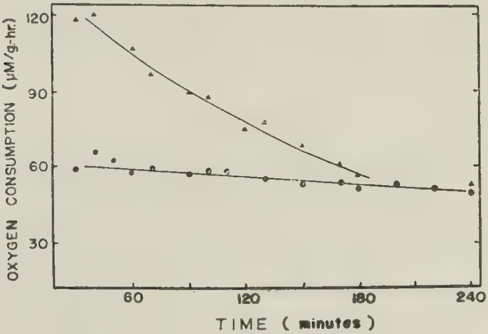


FIG. 3. Oxygen uptake rate of brain slices in the presence (—▲—) or in the absence (—●—) of KCl (10^{-1} *M*). Guinea pig brain slices weighing about 200 mg. were incubated under pure oxygen in 2 ml. of Krebs-Ringer solution containing glucose.

Time Course of Potassium Effect—Fig. 2 shows the time course of the incorporation of P^{32} into protein-bound P in glucose-containing medium with or without high potassium ions. The rate of increase of specific activity was linear with time to 60 minutes and then became a steady state for 240 minutes at 37°. On the other hand, the increase of oxygen consumption by the addition of potassium ions became gradually smaller with time, and after 180 minutes it was almost the same as that in the control medium.

Basing on the results mentioned above, the incorporation of P^{32} into phosphoprotein was usually determined at the end of 90 minute incubation at 37°.

Potassium and Sodium Ions—The effect of an increase in the concentration of potassium ions from 0 to $127\times10^{-3}\,M$ on the incorporation of P^{32} into protein-bound P was examined under the correct osmotic pressure maintained by decreasing the sodium ion concentration substitutionally. As shown in Table III, the presence of certain amount of NaCl was necessary for the increase of the incorporation of P^{32} into protein-bound P by high concentration of potassium ions. On the other hand, the omission of sodium ions being replaced by choline caused much decrease in the P^{32} incorporation. When the concentration of sodium ions was lowered, the incorporation of

TABLE III

Effect of Changing the Concentration of K^+ and Na^+ of the Basic Saline on the Specific Activity of the Protein-Bound P of Guinea Pig Brain Slices

Ion concentration ($10^{-3}\,M$)		Specific activity of protein-bound P (*c.p.m./μg.* P)							Changes (%)
K^+	Na^+	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	
5.1	127	2780	3330	2170	780	1990	1460	1150	(Normal control)
100	127	3640	4960	—	—	4420	2420	—	+67.2 (4)
90	30	—	—	1160, 1550	634, 627	—	—	—	−28.5 (4)
50	70	2740, 4780	—	—	—	—	—	1025, 1085	+16 (4)
5.1	227	—	3510, 3790	1770	—	—	—	—	− 1 (3)
0	127	—	—	3040	—	1680	1320	—	+ 4.5 (3)
5.1	0	—	—	—	392, 146	1170	—	—	−57.7 (3)

Brain slices incubated for 90 minutes at 37° in the medium containing glucose and P^{32}. K^+ was added as KCl and Na^+ was replaced by choline for maintaining the correct osmosic pressure.

TABLE IV

Effect of Adding NH_4^+ or Omitting Ca^{++} on the Specific Activity of the Protein-Bound P of Guinea Pig Brain Slices

Condition	Specific activity of protein-bound P (*c.p.m./μg*) P						Changes (%)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	
Control medium	1800	1343	269	2080	1460	920	
NH_4^+ ($10^{-3}\,M$) added	975	1330	167	—	1470	—	−22.3 (4)
NH_4^+ ($10^{-2}\,M$) added	589	992	107	995	—	—	−50.4 (4)
Ca^{++}-free	1210	—	—	—	—	942, 790	−15.3 (3)

Incubation condition was same as in Table III. NH^{4+} was added as NH_4Cl, and for Ca^{++}-free medium $CaCl_2$ was only omitted from the basic medium.

P^{32} was markedly decreased even in the presence of high concentration of potassium ions.

Ammonium and Calcium Ions—It has previously been reported that the effect of the addition of ammonium ions or the omission of calcium ions on the oxygen uptake and lactic acid formation is similar to that of the high concentration of potassium ions (*4*, *14*). Table IV shows that the addition of ammonium to give a concentration of 10^{-3} or 10^{-2} *M* causes a reduction in the incorporation of P^{32} into protein-bound P. And the omission of calcium ions from the medium also depressed the incorporation of P^{32} slightly.

L-*Glutamic Acid*—Findlay, Magee and Rossiter (*10*) showed that the addition of L-glutamate to the medium has an inhibitory effect on the incorporation of P^{32} into various phosphoric esters of brain slices metabolizing glucose as substrate. It was certainly confirmed as seen in Table V. But when 10^{-1} *M* of KCl was further added to the medium in the presence of glutamate, the specific activity of protein-bound P was increased more than that in the normal control medium. And also, in the case of the addition of fumarate (10^{-2} *M*) to the medium containing glutamate and glucose, the inhibitory effect of glutamate on P^{32} incorporation was considerably abolished.

TABLE V

Effect of the Addition of L-*Glutamate on the Specific Activity of the Protein-Bound P of Guinea Pig Brain Slices in the Presence or in the Absence of 100^{-1} M KCl*

Addition	Specific activity of protein-bound P (*c.p.m./μg.* P)							Changes (%)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	
Control medium	1435	1050	2310	6750	2890	4250	1560	
L-Glutamate	658	925	578	3440	1334	—	—	−51.6 (5)
L-Glutamate and K^+	1650	686	712	—	—	9950	1820	+39.0 (5)
L-Glutamate and fumarate	1355	1170	635	4360	3412	—	—	−30.5 (5)

Experimental condition was same as in Table III.

Tricarboxylic Acid Cycle Intermediates—As naturally expected, when a tricarboxylic acid cycle intermediate was added to the medium containing glucose, the oxygen uptake of brain slices was increased significantly, although glucose consumption and lactic acid formation were rather decreased (Table VII). Therefore, the incorporation of P^{32} into protein-bound P was examined in the addition of succinate, fumarate or α-ketoglutarate to the usual medium.

As shown in Table VI, the incorporation of P^{32} was scarcely affected by the addition of α-ketoglutarate (10^{-2} *M*). The addition of succinate or fumarate caused a slight increase in the incorporation of P^{32} into protein-bound P. Moreover, the addition of both succinate (10^{-2} *M*) and potassium

(10^{-1} *M*) induced the apparent increase of the specific activity of protein-bound P being accompanied with the enhancement of metabolism.

TABLE VI

Effect of the Addition of Some Tricarboxylic Acid Cycle Member on the Specific Activity of the Protein-Bound P of Guinea Pig Brain Slices Metabolizing Glucose

Addition	Specific activity cf protein-bound (*c.p.m./μg.* P)						Change (%)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	
Normal control	454	545	2080	450	2970	2810	
α-Ketoglutarate	—	—	1940	500	2580	—	−3.0 (3)
Fumarate	818	621	—	500	—	2490	+23.5 (4)
Succinate	675	695	2310	—	2350	2830	+13.2 (5)
Succinate and K^+	1540	—	—	—	3660	4190	+101 (3)

Experimental condition was same as in Table III.

TABLE VII

Oxygen Uptake, Glucose Utilization and Lactic Acid Formation in Respiring Brain Slices by the Addition or by the Omission of Various Substances

Condition	O_2 uptake$^+$ (*μM/g./hr.*)	Glucose utilization$^+$ (*μM/g./hr.*)	Lactate formation$^+$ (*μM/g./hr.*)
Control medium	57.4± 3.7 (12)	18.7±3.2 (9)	16.8±4.1 (9)
α-Ketoglutarate	68.5± 8.7 (3)	13.6±2.6 (3)	9.9±1.0 (3)
Succinate	95.7± 1.4 (3)	13.3±1.5 (3)	11.3±3.6 (3)
Fumarate	69.2± 5.4 (3)	13.7±2.6 (3)	12.4±1.7 (3)
Glutamate	75.8± 3.9 (4)	33.7±1.5 (4)	44.4±1.1 (4)
Glutamate and K^+	129.4±12.2 (3)	45.6±2.2 (3)	52.2±5.4 (4)
Succinate and K^+	118.6±10.6 (4)	41.4±3.6 (4)	45.4±4.4 (4)
*NH_4^+ (10^{-2} *M*)	63.5± 5.5 (7)	24.3±3.2 (7)	23.7±2.6 (7)
*Ca^{++}-free	100.3± 7.5 (4)	31.8±3.1 (4)	33.5±2.6 (4)
*K^+-free	60.1± 5.2 (7)	22.5±1.5 (7)	24.3±3.6 (7)
*K^+ (10^{-1} *M*)	94.4± 8.1 (4)	51.0±4.2 (4)	59.4±5.9 (4)

Brain slices incubated for 90 minutes at 37° under pure oxygen in Krebs-Ringer phosphate solution containing glucose.

* These results have already appeared in previous report (*4*)

$^+$ Mean±Standard deviation. Figure in parentheses, number of experiments.

Effect of Adding Some Substrates and Changing Ionic Constituents on Oxygen Uptake, Lactic Acid Formation and Glucose Consumption—Table VII presents that the addition of some tricarboxylic acid cycle intermediate to the medium containing glucose as a substrate augmented the oxygen uptake of brain slices, although the lactic acid formation and glucose consumption were rather decreased. When L-glutamate was added, all of oxygen uptake, lactic acid formation and glucose consumption were increased markedly. And also these were further increased by the further addition of potassium ions (10^{-1} *M*). Furthermore, as previously reported (*4*), the metabolism of brain slices is influenced by changing the concentration of potassium, calcium or ammonium ions.

From above observations, it seems that the augmentation of P^{32} incorporation into protein-bound P would not be related directly with an increase in the metabolism of brain slices, but only the increase of metabolism which was caused by the existence of high concentration of potassium ions could be specially coupled to the phosphorylation mechanism in brain tissue.

Transamination in Brain Slices—For the purpose to explain the inhibitory effect of L-Glutamate on P^{32} incorporation, the transaminase activity in brain slices metabolizing both glucose and L-glutamate was estimated. L-glutamate is metabolized oxidatively in brain slices, and at the same time aspartic acid was also produced from L-glutamate in the presence of glucose about 5 times more than in the absence of glutamate. Further addition of fumarate (10^{-2} *M*) was without effect on the formation of aspartic acid.

TABLE VIII

Changes of the Contents of Aspartic Acid and γ-Aminobutyric Acid in Respiring Guinea Pig Brain Slices by the Addition of L-Glutamate to the Medium

Addition	Aspartic acid (μM/g.)	γ-Aminobutyric acid (μM/g.)
Control medium	1.19±0.50 (3)	1.30±0.45 (4)
L-Glutamate	6.45±1.60 (5)	2.29±0.55 (5)
L-Glutamate and K^+	3.71±1.47 (3)	2.77±0.73 (3)
L-Glutamate and fumarate	7.51±2.39 (4)	2.12±0.80 (3)
KCl (10^{-1} *M*)	0.83±0.14 (3)	1.02±0.39 (3)
Without incubation*	2.82±1.40 (3)	1.61±0.53 (4)

Brain slices incubated for 1 hour at 37° in pure oxygen. Mean±Standard deviation is presented. Figure in parentheses, number of experiments.

* The value estimated immediately after the preparation of slices.

From this result, the transamination between glutamic acid and oxaloacetic acid was indicated to be active in brain slices. Therefore it is suggested that oxaloacetic acid may be deficient for the full operation of tricarboxylic acid cycle in the presence of L-glutamate. In the presence of high concentration of potassium ions the transaminase activity between glutamic acid and oxaloacetic acid was inhibited to about one half. But in the absence of L-glutamate, potassium ions did not affect the content of aspartic acid. The contents of alanine and γ-aminobutylic acid in brain slices were not changed significantly even in the presence of L-glutamate.

DISCUSSION

The cerebral cortex slices metabolizing glucose are able to synthetize phosphocreatine and glycogen *in vitro* (*22, 23*), and an increase of potassium ion concentration in the medium augments the oxygen uptake, lactic acid formation and glucose utilization, as confirmed by many workers. But the concentration of labile P in brain slices are unable to be maintained under this condition of augmenting metabolism (*2, 8*). Findlay, Magee and Rossiter (*10*) indicated that an increase of potassium ion concentration in the incubating medium causes a decrease in the incorporation of P^{32} into the various phosphoric esters of slices of cat brain as well as the omission of calcium or the addition of ammonium does.

On the contrary the results in this paper show that incorporation of P^{32} into acid-soluble P and protein-bound P of guinea pig brain slices is increased markedly by the addition of excess potassium. Findlay *et al.* (*10*) carried out their experiments under the isotonic potassium medium where most of sodium ions were omitted. In these conditions, the enhancement of metabolism is never observed even when the excess of potassium ions are added, as previously reported (*4, 24*). Anyhow it is necessary for the increase of the incorporation of P^{32} that sodium ions exist together with a requisite quantity of potassium ions (Table III). The hypertonicity by the addition of NaCl has not any particular effect on P^{32} incorporation.

In liver slices, an increase of oxygen uptake was not caused even by the addition of potassium ions (*4*), and also the incorporation of P^{32} into protein-bound P was not affected at all.

From these findings it seems that the augmented metabolism of glucose of brain slices in the presence of excess potassium might be coupled closely with the phosphorylation mechanism, especially with the turnover of phosphoprotein within the slices.

In brain slices the specific activity of phosphoprotein was considerably greater than that of any other P-containing compounds. Similar results have already been reported with brain and liver. This fraction does not contain the nucleotide phosphorus, and the residual phosphorus after the removal of the acid-soluble, lipid and nucleic acid P, can be fractionated by alkaline hydrolysis into an inorganic P (phosphoprotein), an organic fraction and a true residue (*9, 27*). The experiments would indicate that the incorporation of P^{32} into phosphoprotein fraction of brain slices is not of a nonspecific contamination of P^{32}, but of the active incorporation into the protein due to the oxidative metabolism of glucose. The results presented here are also in agreement with the findings of Findlay *et al.* (*9*) and Engelhardt *et al.* (*26*).

Although the chemical nature of phosphoprotein was discussed by many workers (*9, 25*), the precise nature of it in brain tissue is not yet known. Vladimirov and others (*28, 29*) showed that the specific activity of the phosphoprotein in brain tissue was increased under the excitatory state *in* *vivo*, and on the contrary it was strongly depressed at the hibernation. On the other hand, it is thought that the increase of the specific activity of phosphoprotein due to the addition of high potassium ions could be concerned with the sodium extrusion from the cells requiring the much energy expenditure. If much of the metabolism is directed towards the maintenance of a high gradient of potassium ions between the inside and the outside of the cells (*30, 31*), the enhancement of the phosphorylation would be expected for satisfying a greatly increased expenditure of energy. At any rate, the phosphoprotein fraction may represent an important role concerning the physiological activities of brain.

Some of the tricarboxylic acid cycle intermediates increased the oxygen uptake of brain slices, although the incorporation of P^{32} was not affected remarkably. Although L-glutamate which might play an important role in brain tissue (*31, 32*) increases the aerobic metabolism of glucose like the addition of potassium ions, it decreases the contents of phosphocreatine of brain slices, and also inhibits the incorporation of P^{32} into the P-containing compounds when it is added in the presence of an adequate concentration of glucose.

Although a possible explanation of these observations has been presented elsewhere (*10*), another consideration may be possible. Table VII shows that the addition of potassium ions (10^{-1} *M*) to the medium containing both glucose and glutamate increases further the oxygen uptake, lactic acid formation and glucose utilization. This finding does not concur with the results of Lipsett and Crescitelli (*33*). The inhibitory effect of L-glutamate on the energy yielding process is thought to be explained by the possibility that the increase of aspartic acid production in the presence of glutamate, which is caused by the transamination between glutamic acid and oxaloacetic acid, may induce the insufficiency of the operation of the tricarboxylic acid cycle, consequently of the oxidative metabolism of glucose. These thoughts may be supported by the fact that the inhibitory effect of glutamate on the incorporation of P^{32} is depressed by the addition of excess potassium ions or fumarate to the medium containing both glucose and glutamate, since this addition should result in recovering a loss of oxaloacetic acid due to transamination.

SUMMARY

1. The enhancement of metabolism of guinea pig brain slices respiring in Krebs-Ringer glycylglycine buffer solution by the addition of KCl (10^{-1} *M*) was accompanied with an increase in the incorporation of P^{32} phosphate into the protein-bound P, especially into "phosphoprotein" P, of them. These results were peculiar to the brain slices.

2. It was quite necessary for an increase of P^{32} incorporation into the protein-bound P that the high concentration of potassium ions is added to the usual Krebs-Ringer solution without reducing sodium concentration. The hypertonicity, which was caused by the addition of NaCl, had no effect

on P^{32} incorporation. The omission of sodium ions from the medium being replaced by choline caused the remarkable decrease of P^{32} incorporation, but the omission of potassium ions had no effect.

3. The addition of L-glutamate to the medium in the presence of glucose inhibited P^{32} incorporation into the protein-bound P, but the further addition of KCl (10^{-1} *M*) caused a more increase in the incorporation of P^{32} than the normal control medium. In the case of the further addition of fumarate to the medium containing glucose and L-glutamate, the inhibitory effect of L-glutamate was rather diminished. By the addition of L-glutamate to the medium metabolizing glucose, the content of aspartic acid in the slices was increased apparently and that of γ-aminobutyric acid was not changed significantly, so the oxaloacetic acid might become insufficient for the operation of tricarboxylic acid cycle.

4. The incoporation of P^{32} into protein-bound P was not changed significantly even by the addition of some tricarboxylic acid cycle intermediate to the usual medium, although in the further addition of KCl (10^{-1} *M*) it was increased.

5. The effect of changing the ionic constituents of medium or adding some metabolite on the oxygen uptake, aerobic lactic acid formation and glucose utilization were also studied. And the relationships between the metabolic patterns and the incorporation of P^{32} in the cerebral cortex slices were discussed.

REFERENCES

(*1*) Tsukada, Y., and Takagaki, G., *Nature*, **175**, 725 (1955)
(*2*) Tsukada, Y., and Takagaki, G., *Symposia on Enzyme Chemistry*, (*in Japanese*) **10**, 165 (1954)
(*3*) Takagaki, G., Hirano, S., and Tsukada, Y., *Arch. Biochem. Biophys.*, **68**, 196 (1957)
(4) Takagaki, G., Tsukada, Y., J. Neurochem., **2**, 21 (1957)
(*5*) Ashford, G., and Dixon, K. C., *Biochem. J.*, **29**, 157 (1935)
(*6*) Dickens, F., and Greville, G. D., *Biochem. J.*, **29**, 1468 (1935)
(*7*) Weil-Malherbe, H., *Biochem. J.*, **32**, 2257 (1938)
(*8*) Heald, P. J., *Biochem. J.*, **57**, 673 (1954)
(*9*) Findlay, M., Strickland, K. P., and Rossiter, R. J., *Canad. J. Biochem. Physiol.*, **32**, 504 (1954)
(*10*) Findlay, M., Magee, W. L., and Rossiter, R. J., *Biochem. J.*, **58**, 236 (1954)
(*11*) Deluca, H. A., Rossiter, R. P., and Strickland, K. P., *Biochem. J.*, **55**, 193 (1953)
(*12*) Strickland, K. P., *Canad. J. Biochem. Physiol.*, **32**, 50 (1954)
(*13*) Takagaki, G., Hirano, S., Sugimoto, S., and Tsukada, Y., *Symposia on Enzyme Chemistry* (*in Japanese*) **11**, 1 (1956)
(*14*) Gore, M. B. R., and McIlwain, H., *J. Physiol.*, **117**, 471 (1952)
(*15*) Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, **177**, 775 (1949)
(*16*) Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21**, 465 (1949)
(*17*) Koehler, L. H., *Anal. Chem.*, **24**, 1576 (1952)
(*18*) Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941)
(*19*) Awapara, J., Landua, A. J., and Fuerst, R., *Biochem. Biophys. Acta*, 5, 457 (1950)
(*20*) Yemm, E. W., and Cocking, E. C., *Analyst*, **80**, 209 (1955)

(21) Kimura, Y. and Niwa, T., *Nature*, **171**, 881 (1953)
(22) McIlwain, H., Buchel, L., and Cheshire, J. D., *Biochem. J.*, **48**, 12 (1951)
(23) LeBaron, F. N., *Biochem J.*, **61**, 80 (1955)
(24) Canzanelli, A., Rogers, G., and Rapport, F., *Am. J. Physiol.*, **135**, 309 (1942)
(25) Davidson, J. N., Frazer, S. C., and Hutchison, W. C., *Biochem. J.*, **49**, 311 (1951)
(26) Engelhardt, V. A., et Lissovskaia, N. P., Communication au XIX^e^ Congres International de Physiologie, p. 216 (1953)
(27) Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945)
(28) Vladimirov, G. E., Communication at the XIX International Physiological Congress, Acad. Sci. U.S.S.R., p. 40 (1953)
(29) Skvirskaya, E., and Silich, T., Biochemistry of the Nervous System, Kiev, p. 36, (1954)
(30) Hodgkin, A. L., and Keynes, R. D., *Symp. Soc. Exp. Biol.*, **8**, 423 (1954)
(31) Terner, C., Eggleston, L. V., and Krebs, H. A., *Biochem. J.*, **47**, 136 (1950)
(32) Weil-Malherbe, H., *Physiol. Rev.*, **30**, 549 (1950)
(33) Lipsett, M. N., and Crescitelli, F., *Arch. Biochem.*, **28**, 329 (1950)

ADDENDA

After the authors had completed the manuscript of this paper, two reports by Heald were published (Heald, P. J., *Biochem. J.*, **66**, 659, and **67**, 529, (1957)). It was shown that the " phosphoprotein " fraction was implicated in the rapid phosphate-exchange reactions promoted by applied electrical pulses *in vitro.* He assumed the " phosphoprotein " fraction to play a very important role in metabolism and also in function of cerebral tissues, while the precise nature of " phosphoprotein " was not put forward. Also, he reported that the specific radioactivity of guanosine di- and tri-phosphates was increased upon the passage of electrical pulses through the slices. It is the authors' thought that the findings by Heald may be related in some aspects to the present observations, though the characteristics of the potassium effect are very different from the electrical stimulation studied by Heald. Investigations with a finer technique of fractionation than the present one may be intended in future.

The Journal of Biochemistry, Vol. 45, No. 7, 1958

CRYSTALLINE BACTERIAL PROTEINASE

V. ON THE REACTION WITH DIISOPROPYL FLUOROPHOSPHATE

BY HIROSHI MATSUBARA AND SHIGEHIKO NISHIMURA

(From the Department of Biology, Faculty of Science,
University of Osaka, Osaka)

(Received for publication, October 2, 1957)

In previous reports it has been shown that *Bac. subtilis N′* proteinase (BPN′) has the esterase activity (*1*) and consists of a single polypeptide chain having alanine as the amino-terminal residue in both experiments of treating BPN′ with trichloroacetic acid solution and diisopropyl fluorophosphate (DFP) (*2*). As DFP is a typical inhibitor acting on chymotrypsin and trypsin, it is of interest to investigate whether DFP reacts with BPN′ or not.

It has been found by Jansen *et al.* (*3*) that the esterase and proteinase activities of α-chymotrypsin and trypsin are simultaneously inhibited by DFP, suggesting that both activities occur from the same active center in an enzyme protein. The diisopropyl phosphoryl (DIP) derivatives of α-chymotrypsin (*3*), β- and γ-chymotrypsin, and trypsin (*4*) have been isolated in crystalline form, contributing to the investigation of the relation between structure and activity of the proteolytic enzymes. Besides such animal and neutral proteolytic enzymes, a proteolytic enzyme of *Bac. subtilis*, " subtilisin ", was inhibited by DFP (*5, 6*). It has also been found by the present authors that the esterase and proteinase activities of BPN′ were simultaneously inhibited by DFP and the inhibited compound was isolated in a stable crystalline form.

Furthermore, since the behavior of proteolytic enzyme towards some of protein inhibitors would offer a clue in solving some of the important questions, reactions of BPN′ with soy bean-trypsin inhibitor, which is a trypical naturally occurring trypsin inhibitor found by Kunitz (*7, 8*), and some inhibitors from other source materials were examined, and then it was found that BPN′ was completely inhibited by crude extracts from potato and broad bean, as reported by Matsushima (*9*), but there is no decrease in the activity of BPN′ in the presence of soy bean-trypsin inhibitor under various conditions. These facts are highly interesting in the investigation of the active area of BPN′ compared with the other proteolytic enzymes. The present paper reports the results of experiments designed to determine the question of whether the enzyme BPN′, though it has a broad specificity against trypsin (*10*), can be essentially distinguished from trypsin or not.

MATERIALS AND METHOD

BPN′* recrystallized twice (*1*, *11*) was used after washing with 45 per cent acetone. The protein concentration was determined from the optical density at 280 mμ in which the conversion factor to protein mg. per ml. was about 0.88.

Trypsin used was a commercial sample from the Mochida Pharm.

Diisopropyl fluorophosphate was kindly supplied by Mr. T. Ikenaka of Akabori Laboratory.

Crystalline soy bean-trypsin inhibitor was obtained according to the method of Kunitz (*8*), and its crude material was obtained by extracting soy bean with 0.25 N H_2SO_4 after ethanol washing.

The crude extracts from potato and broad bean were prepared according to the method reported by Matsushima (*9*) as follows: *a*) *potato*—Thirty g. of washed potato was breied and homogenized with 30 ml. of 0.1 N HCl containing 1 per cent NaCl for 1 hour at room temperature. Starch was separated by decantation and the supernatant solution was heated at 65° for 5 minutes and cooled rapidly. After centrifugation, the supernatant solution was used as the crue material. *b*) *broad bean*—Thirty g. of the powder was extracted with 1 per cent NaCl for 1 hour at 30° occasionally stirring, and the suspension was filtered. The filtrate was boiled at 80° for 3 minutes and rapidly cooled, after adjusting the pH to 2.5 with N HCl. The resulting colloidal suspension was filtered, and the filtrate was used as the crude material.

Assay methods for proteinase and esterase activities were reported previously (*1*, *11*), in which casein was used as a substrate for the former and methyl butyrate for the latter.

Inhibition experiments by the crude materials were carried out as follows: After 1 ml. of about 0.001 per cent BPN′ solution was preincubated with 2 ml. of the extracts previously adjusted the pH to 7.0 with $M/2$ Na_2HPO_4 for 10 minutes at 30°, the residual proteolytic activity was measured according to the method described in previous paper (*1*). Control was also tested without inhibitors. The method of inhibition experiment with crytalline soy bean-trypsin inhibitor and DFP was similar to that mentioned above and described in each section of results.

RESULTS

Inhibition of Esterase and Proteinase Activities of BPN′ by DFP—The reaction with DFP was carried out in the following manner: A stock solution of 10^{-1} M DFP was prepared by dissolving in anhydrous isopropanol, and from this stock solution of DFP, solutions of desired concentrations containing 10 per cent of isopropanol were prepared and used within 2 hours. Two ml. of DFP solution of each concentration ranging from 10^{-3} to 10^{-6} M was reacted with 2 ml. of enzyme solution (about 3 mg. per ml.) at pH 7.5 to 8.0** for 30 minutes at 30°. An aliquot was removed at intervals and the esterase activity was assayed and the proteinase activity after dilution about 100 times with $M/20$ phosphate buffer of pH 8.0. The proteinase activity of BPN′ was completely inhibited by excess DFP within 5 minutes at pH 7.5, in which the enzyme was very stable (*1*), similar to α-chymotrypsin (*3*).

---

* Crystalline material was kindly supplied by Nagase & Co. Ltd.

** The reaction proceeded in the same manner at pH 7.0 to 8.5.

Although it was sufficient to react BPN′ with DFP during a short time, it was allowed to stand for 30 minutes in the following experiments in order to be certain.

The effect of various concentrations of DFP on both activities of BPN′ is shown in Fig. 1; like the result obtained by Jansen *et al.* (*3*) it was shown that the esterase and proteinase activities decreased simultaneously with concentration of the inhibitor, the concentration which caused 50 per cent inhibition being about 2.96×10^{-5} *M*, and so the ratio of DFP combined with BPN′ was 1.13, assuming the molecular weight of BPN′ to be 30,500 (*2*).

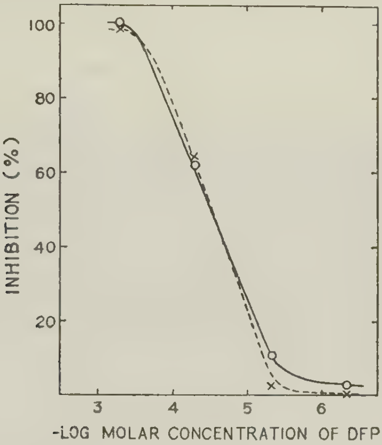


FIG. 1. Inhibition of the protoeinase and esterase activities ofBPN′ by DFP.

The concentration of BPN′ was 1.6 mg. protein per ml. of *M*/20 phosphate buffer of pH 8.0. The reaction time was 30 minutes at 30°. --×--: esterase activity, —○—: proteinase activity.

Crystallization of DIP-BPN′—After about 1 g. of twice recrystallized BPN′ was washed with 45 per cent acetone, it was dissolved in 50 ml. of 0.01 *M* sodium acetate of pH 8.0, and freshly prepared, excess DFP (20 to 50 fold) dissolved in acetone was added to this enzyme solution. After standing for 30 minutes at 30°, adjusting the pH to 8.0 with *N* NaOH, excess acetone was added to the reaction mixture and the DIP-BPN′ complex was collected by centrifugation. The precipitate was dissolved in a small volume of distilled water and acetone was gradually added to the solution until it becomes turbid slightly (about 35 to 40 per cent acetone in final). Gelatinous substance sticked on the wall of glass tube, which could be dissolved in acidic condition, was removed and standing in a refrigerator for one to several hours, and then large rod crystals were obtained. Recrystallization was done by the following procedure: The crystals collected was dissolved by adjusting the pH to 9.5 with *N* NaOH and after centrifugation the pH of the solution was adjusted to 7.0 with N CH_3COOH or HCl, and, if necessary, acetone was added. Thus the crystals were repeatedly obtained. When the crystals were washed with acetone (40 per cent) or dialyzed against distilled water, residual DFP was almost removed. The photograph (Fig. 2) shows the crystals of DIP-BPN′.

Some Propertis of DIP-BPN′—(a) As shown in the photograph, crystals of DIP-BPN′ are similar to those of BPN′ slowly grown in a refrigerator, but, its solubility is lower than that of BPN′, namely DIP-BPN′ is not dissolved by 30 per cent acetone. (b) DIP-BPN′ recrystallized three times had a proteinase activity of about 0.14 per cent of the original active proteinase at pH 7.5 and 0.17 per cent at pH 10.0, similar to the other proteolytic enzymes (*12–14*). (c) In order to find if the DIP-derivative becomes susceptible to the original active proteinase, a following experiment was carried out. Five ml. of about 0.05 per cent BPN′ was added to 10 ml. of about 0.14 per cent DIP-BPN′ solution (pH 8.2) dissolved in $M/20$ phosphate buffer at 30° (the final ratio of DIP-BPN′ to BPN′ was $5:1$ w/w) and at intervals, an aliquot was removed and precipitated with $1.2\,M$ trichloroacetic acid solution to terminate the enzyme action. The absorption of the filtrate of the precipitated solution at $280\,m\mu$ was determined and DIP-BPN′ was found to be not affected at all by BPN′ after incubation for 120 minutes as shown in Fig. 3. On the contrary, DIP-BPN′ previously heated at 90° for 30 minutes was digested rapidly by BPN′ within several minutes. This result showed that BPN′ does not become susceptible even after modification by DFP. (d) Electrophoretic behavior showed the homogeneity of DIP-BPN′ at pH 7.5 and 8.0, and it migrated towards the anode. (e) Absorption spectrum of DIP-BPN′ was similar to that of BPN′. (f) The molecular weight of DIP-BPN′, was about 31,000 to 32,000 by monomolecular film measurements* (*cf.* The molecular weight of BPN′ was

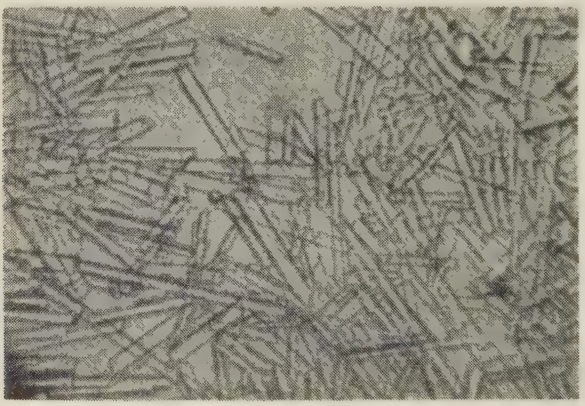


FIG. 2. Crystalline DIP-BPN′ (×2400)

---

* We are indepted to Mr. Ikeda of Isemura Laboratory for the measurement of molecular weight of DIP-BPN′ and of BPN′.

assumed to be 30,500 or 31,300 (*2*).). (g) The amino-terminal amino acid residue was one mole of alanine (*2*).

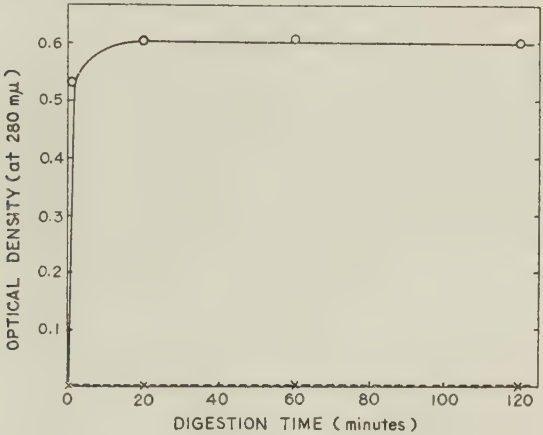


FIG. 2. Digestion course of DIP-BPN′ treated with and without heat by BPN′.

Five ml. of 0.05 per cent BPN′ solution dissolved in $M/20$ phosphate buffer of PH 8.0 was added to 10 ml. of 0.14 per cent DIP-BPN′ solution treated with and without heat (90° for 30 minutes) and at intervals the optical density of soluble material in concentrated TCA solution was measured at 280 mμ. —○—: treated, --×--: non-treated.

TABLE I

Inhibition of BPN′ by Some Crude Extracts

Inhibitor	Soy bean		Broad bean		Potato	
	+	−	+	−	+	−
Activity of BPN′*	0.048	0.061	0.002	0.058	0.000	0.064
Inhibition (%)	21		97		100	

* Optical density at 660 mμ corrected for blank test.

Reaction of BPN′ and Soy Bean-Trypsin Inhibitor—Trypsin activity was tested during the over-all process of this inhibition experiments for reference. Although trypsin was approximately inhibited by this inhibitor, BPN′ was not inhibited even when a large excess of the inhibitor was applied in the pH range of 6 to 8.5. Examination was made to elucidate whether competition between substrate and inhibitor was present or not when the substrate concentration was lowered, but no inhibition was found. Accordingly it was concluded that BPN′ was not inhibited by the soy bean-trypsin inhibitor.

Reactions of BPN′ and Crude Extracts of Potato, Broad Bean, and Soy Bean.—As shown in Table I BPN′ was not particularly inhibited by the soy bean extract, but strongly inhibited by the extracts from potato and broad bean.

DISCUSSION

It was found that both esterase and proteinase activities of BPN′ were simultaneously inhibited by DFP, similar to the result of chymotrypsin and trypsin (*3*), suggesting that the activities might be derived from an active area of the enzyme protein. However, crystalline soy bean-trypsin inhibitor did not inhibit the proteinase activity of BPN′. On the contrary, this enzyme was strongly inhibited by the extracts, though crude, from potato and broad bean, as also shown by Matsushima (*9*). In Table II the relationship between some inhibitors and two proteolytic enzymes BPN′ and trypsin, was roughly shown. It may be considered that trypsin inhibitor and BPN′ inhibitor from soy bean are two different substances and its trypsin inhibitor acts specifically on trypsin. A similar consideration may be applied on the broad bean extract. Recently Sohonie and Ambe (*15*) have crystallized two kinds of trypsin inhibitor from potato and it must be interesting to compare the inhibition feature on trypsin and BPN′. Additionally, considering the fact that BPN′ has a broad specificity (*10*) and behavior towards the inhibitors, there may be an interesting relation between structure and activity, involving specificity, of the proteolytic enzyme, Since DFP uniformly inhibits the activity of some proteolytic enzyme, such as chymotrypsin, trypsin and bacterial proteinases, which have different specificities and structures, it is considered that at least the inhibitor, DFP, does not react with the groups determining the specificity of the enzyme, but with some groups that are directly necessary for the activation of the peptide or ester bond and that are common to all those enzymes, as suggested previously (*16*, *17*), although the reaction between inhibitor and enzyme might be a different one from the reaction between substrate and enzyme.

TABLE II

Degree of Inhibition of BPN′ and Trypsin by Some Naturally Occurring Inhibitors

Inhibitor	Trypsin	BPN′
Crystalline soy bean	⧻	−
Crude soy bean	⧻	+
Crude broad bean	+ (*9*)	⧻
Crude potato	⧻ (*9*)	⧻

According to the analysis of DIP-derivatives of chymotrypsins and trypsin (*12*) they contain one mole of phosphorus and two alkyl groups per protein molecule, but in the case of BPN′ described in this paper no chemical analysis was made, and accordingly, it is uncertain whether BPN′ reacts with DFP in the molar ratio of one to one or more. Chemical anlyses of phosphorus and alkyl groups contained in DIP-BPN′ and of the amino acids or peptides in the protein combining with DIP-radical will be carried out in the future.

The fact that the soy bean-trypsin inhibitor did not react with BPN′ may suggest that the inhibitor has a high specificity and BPN′ has a different structure in the region of active area from trypsin, and moreover, DFP, a small molecule, must react at least with a certain common site at the active area of the proteolytic enzymes.

The gelatinous substance removed on purification of DIP-BPN′ may be some peptides binding with BPN′ as suggested on the reaction of DFP with chymotrypsin (*18*).

The fact that DIP-BPN′ was not digested by BPN′, measuring the absorption at 280 mμ, shows that BPN′ was not affected by modification with DFP in regard to its susceptibility to BPN′ and so it will be a native protein (*19*).

SUMMARY

The proteinase and esterase activities of bacterial proteinase were simultaneously inhibited by diisopropyl fluorophosphate, and the diisopropyl phophoryl derivative was obtained in a stable crystalline form. Using the proteinase method it was also confirmed that the secondary structure of bacterial proteinase is never altered by its reaction with diisopropyl fluorophosphate, and some properties of the derivative were shown. Contrary to trypsin, the bacterial proteinase was not inhibited under various conditions. However, this proteinase was strongly inhibited by the crude extracts from potato and broad bean. Then, some discussions were made on the relation between inhibitor and enzyme.

The authors wish to express their many thanks to Prof. K. Okunuki and Dr. B. Hagihara for their kind advices through this work, to Mr. T. Ikenaka for his gift of DFP, and also to Nagase & Co., Ltd. for the gift of crystalline bacterial proteinase.

REFERENCES

(*1*) Matsubara, H., Hagihara. B., Nakai, M., Komaki, T., and Okunuki, K., *J. Biochem.*, **45**, 251 (1958)

(*2*) Matsubara, H., and Nishimura, S., *J. Biochem.*, **45**, 413 (1958)

(*3*) Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.*, **176**, 189 (1949)

(*4*) Jansen, E. F., and Balls, A. K., *J. Biol. Chem.*, **194**, 721 (1952)

(*5*) Güntelberg, A. V., and Ottesen, M., *Compt. -rend. trav. lab. Carlsberg. Sér. Chim.*, **29** (No. 4), 36 (1954)

(*6*) Steinberg, D., *J. Am. Chem. Soc.*, **75**, 4875 (7953)

(*7*) Kunitz, M., *Science*, **101**, 668 (1945)

(*8*) Kunitz, M., *J. Gen. Physiol.*, **29**, 149 (1946)

(*9*) Matsushima, K., *J. Agr. Chem. Soc. Japan* (*in Japanese*), **31**, 38 (1957)

(*10*) Okunuki, K., Matsubara, H., Nishimura, S., and Hagihara, B., *J. Biochem.* **43**, 857 (1956)

(*11*) Hagihara, B., *Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954)

(*12*) Balls, A. K., and Jansen, E. F., *Adv. in Enzymol.*, **13**, 321 (1952)

(*13*) Gladner, J. A., and Neurath, H., *J. Biol. Chem.*, **206**, 911 (1954)

(*14*) Gladner, J. A., and Neurath, H., *Biochim. Biophys. Acta.* **9**, 335 (1952)

(*15*) Sohonie, K., and Ambe, K. S., *Nature* (London), **176**, 972 (1955)

(*16*) Green, N. M., *J. Biol. Chem.*, **205**, 535 (1953)

(*17*) Green, N. M., and Neurath, H., The Proteins, ed. by Neurath, H., and Bailey, K., Academic Press INC., Publishers, New York, Vol. **II**, Part **B**, p. 1057 (1954)

(*18*) Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.*, **185**, 209 (1950)

(*19*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem.* **43**, 453 (1956)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

STUDIES ON THE RIBONUCLEASE-RESISTANT FRACTION OF THE RIBONUCLEIC ACID FROM TOBACCO MOSAIC VIRUS

By KENTARO TANAKA* and TADAO USAMI

(From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya and the Department of Plant Pathology, Faculty of Agriculture, Nagoya University, Anjo)

(Received for publication, December 23, 1957)

The base composition of the ribonucleic acid (RNA) from tobacco mosaic virus (TMV) has been very extensively investigated (*1–3*). It has been shown that the content of adenine is higher than that of guanine. Recently, Raddi and Knight (*4*) reported on the base ratio of trichloroacetic acid-precipitable material after pancreatic ribonuclease (RNase) digestion of TMV nucleic acid. It is clear from their results that the RNase-resistant residue of the TMV-RNA is more abundant in adenine than the original TMV-RNA, in contrast to the cases of RNA from pig liver and yeast (*5–6*).

This fact appears to provide a useful means for the elucidation of the structural difference among these RNAs. In the present studies, the base ratios of the nondialyzable materials in the RNase digests of TMV and yeast RNAs were determined. It was thus found that the action of RNase on these two RNAs produces resistant fractions which are remarkably richer in adenine (TMV-RNA) or guanine (yeast RNA) than their original RNAs.

MATERIALS AND METHODS

Pancreatic RNase—The enzyme used in the study was a crystalline preparation obtained from Worthington Biochemical Sales Corporation.

Ribonucleic Acid from Tobacco Mosaic Virus (*TMV-RNA*)—TMV (ordinary strain) was isolated from infected tobacco leaves by fractional centrifugation according to the method of Knight and Stanley (*7*). The nucleic acid of the virus was detached from protein by heating for 1 minute as described by Knight (*1*) and purified by acid precipitation. (P, 7.8 per cent)

Yeast Ribonucleic Acid—Commercial yeast RNA (sodium salt, Schwarz). (P, 8.3 per cent)

Digestion with RNase—14 mg. of TMV-RNA were dissolved in 2 ml. of 0.25 *M* Tris buffer of pH 7.6. Since a decrease in the pH was caused when the RNA went into solution, the pH was carefully readjusted by the addition of *N* NaOH. To the solution, 56 μg. of crystalline RNase in 0.5 ml. of the same buffer were mixed and incubated at 37° for 18 hours. After incubation, the mixture was dialyzed in a cellophane tubing against

---

* Present adress: Shionogi Research Laboratory Shionogi Co., Ltd. 192 Imafuku, Amagasaki-shi Hyogo Pref., Japan.

3 liters of cold distilled water overnight with three changes of water.

50 g. of sodium salt of yeast RNA were dissolved in 500 ml. of distilled water and deproteinised by chloroform gel formation and exhaustively dialyzed. The inside liquid (concentrated to 250 ml.) was digested with 8.5 mg. of crystalline RNase at 37° for 18 hours with frequent addition of NaOH to maintain the pH at 7.2. After incubation, the reaction mixture was throughly dialyzed in a cellophane tubing against distilled water and the inside liquid was lyophilized.

Determination of Base Ratio—This was carried out by the method of Smith and Markham (*8*) with a solvent system composed of *tert.*-butanol and HCl.

RESULTS AND DISCUSSION

Table I and Fig. 1 show the base ratios of TMV- and yeast RNA before and after RNase digestion. It can be seen that TMV-RNA has higher content of adenine than yeast RNA, and that a remarkable increase in the adenine content takes place after the digestion of TMV-RNA. Yeast RNA, on the other hand, is rich in guanine and the guanine content increases after the digestion.

The results described above also show that the adenine content of our RNase-resistant fraction obtained by dialysis is higher than that of the TCA-precipitable portion reported by Knight (*4*). From the comparison of Pu/Py ratios of the both preparations, it is indicated that our sample has a smaller average molecular weight than that of Knight. It does not seem unreasonable from these facts to consider that the adenylic acid residues in TMV-RNA are unequally distributed and its considerable portions is concentrated in nondialyzable oligonucleotide fractions which resist the RNase digestion. In yeast RNA, on the other hand, the guanylic acid residues are considerably concentrated in larger molecular fractions obtained by RNase digestion of the original RNA.

TABLE I

The Ratios of the Bases Found in Nucleic Acids from TMV and Yeast before and after RNase Digestion

Sample	Base ratio*				Pu/Py
	A	G	U	C	
TMV-RNA					
Before RNase digestion	10	8.7	10.1	6.7	1.1
RNase resistant material	10	5.6	2.8	4.5	2.2
Yeast-RNA					
Before RNase digestion	10	12.5	9.2	8.7	1.3
RNase resistant material	10	17.8	4.2	4.5	3.2

* A: Adenine, G: Guanine, U: Uracil, C: Cytosine.

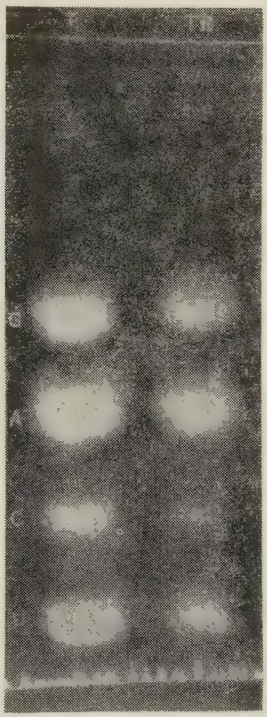


FIG. 1. Chromatographic detection of the bases.

Each sample was hydrolyzed with 1 *N* HCl for 1 hour at 100° and chromatographed using *tert.*-butanol-HCl-H_2O as a solvent.

T: TMV-RNA.
TR: RNase resistant material of TMV-RNA.
A: Adenine.
G: Guanine.
C: Cytidylic acid.
U: Uridylic acid.

SUMMARY

The base ratios of yeast and TMV-RNA were compared with those of their nondialyzable RNase-resistant material. Remarkable increase in the proportion of adenine was observed in the case of RNase digestion of TMV-RNA, while RNase-resistant material from yeast RNA had a higher guanine content than its starting material.

This work was supported by a grant from the Scientific Research Fund of the Ministry of Education.

The authors wish to express their gratitude to Prof. F. Egami for his encouraging interest in this investigation.

REFERENCES

(*1*) Knight, C. A., *J. Biol. Chem.*, **197**, 241 (1952)
(*2*) Markham, R., and Smith, J. D., *Biochem. J.*, **46**, 513 (1950)
(*3*) Cooper, W. D., and Loring, H. S., *J. Biol. Chem.*, **211**, 505 (1954)
(*4*) Reddi, K. K., and Knight, C. A., *J. Biol. Chem.*, **221**, 629 (1956)
(*5*) Magasanik, B., and Chargaff, E., *Biochem. et Biophys. Acta*, **7**, 396 (1951)
(*6*) Volkin, E., and Cohn, W. E., *J. Biol. Chem.*, **205**, 767 (1953)
(*7*) Knight, C. A., and Stanley, W. M., *J. Biol. Chem.*, **141**, 29 (1941)
(*8*) Smith, J. D., and Markham, R., *Biochem. J.*, **46**, 509 (1950)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

FURTHER STUDIES ON PHENOL OXIDASE RELATED TO THE LIGNIN BIOSYNTHESIS

By TAKAYOSHI HIGUCHI

(*From the Laboratory of Wood Chemistry, Faculty of Agriculture, University of Gifu, Naka, Gifu*)

(Received for publication, April 16, 1958)

It has been reported by Freudenberg *et al.* (*1*) that coniferous lignin is formed from coniferyl alcohol under the influence of mushroom "phenodehydrogenase" and oxygen. In "The Enzymes" edited by Sumner and Myrbäck, descriptions of the phenoldehydrogenase are also found (*2*), but the properties of the enzyme have not yet been characterized.

Mason and Cronyn (*3*) confirmed recently that the oxidation of coniferyl alcohol was catalyzed by crude mushroom extracts, but the purified mushroom polyphenol oxidase (tyrosinase) the oxygen consumption was negligible. In previous papers, dealing with laccase of Japanese lacqueur and wild mushroom (*Lactarius piperatus*), the present author (*4*, *5*) reported that Freudenberg's phenoldehydrogenase might probably be a laccase.

Further experimental results on this problem, using a variety of phenylpropane compounds related to lignin as substrates, are described in the present report.

MATERIALS AND METHODS

Mushroom (*Psalliota campestris*) *Phenol Oxidase*—Fruit bodies of the mushroom cultivated at Sekigahara, Fuwa District, Gifu prefecture, were collected on the morning of May 16, 1957. In the afternoon of that day, fresh fruit bodies (about 800 g.) were homogenized with some amounts of cold phosphate buffer (1/15 *M*, pH 6.8) in a Waring blendor. The homogenate was squeezed through lint, and about 1 liter of a brown extract was obtained. Further steps of the preparation method from this brown solution were the same as those described in Freudenberg's report (*6*).

Laccase of Japanese Lacqueur—According to the method of Yakushiji (*7*), 2 liters of cold acetone was added under stirring to about 800 g. of commercial Japanese lacqueur. The brown colored acetone solution was filtered, and the sediment was well washed with cold acetone until the filtrate became colorless. The sediment was extracted overnight with 1.5 liter of distilled water in a refrigerator. The extract containing the laccase was filtered on a Buchner funnel, and 750 g. of solid $(NH_4)_2SO_4$ was added to the filtrate (about 1.5 liter, blue colored solution) under continuous stirring. The precipitate was centrifuged off, and then solid $(NH_4)_2SO_4$ was added to the supernatant at 40° until saturation. The blue precipitate was collected by centrifugation and was dissolved in small amounts of distilled water. This was used as an enzyme solution after dialysis.

Potato Tyrosinase—The outer part of the potato tuber, abut 1 cm. thick, was homogenized with a small amount of distilled water in a Waring blendor, and about 500 g. of the homogenate was obtained. The homogenate was extracted by 250 ml. of cold distilled water. Then, 150 ml· of cold acetone was added to the extract under continuous stirring. The precipitate was centrifuged off, and further 200 ml. of cold acetone was added. The precipitate was collected by centrifugation and was dissolved in 25 ml. of phosphate buffer (1/15 *M*, pH 6.8). This was used as an enzyme solution (*8*).

Substrates—α-Hydroxypropiovanillone, guaiacylacetone and vanilloyl methyl ketone were synthesized according to the method of Hibbert (*9–11*), and *p*-hydroxycinnamic acid, caffeic acid, ferulic acid, and synap acid were synthesized by condensing *p*-hydroxybenzaldehyde, protocatechuic aldehyde, vanillin, and syringic aldehyde, respectively, with malonic acid according to the method of Kratzl (*12*). Coniferyl alcohol and guaiacylglycerol were prepared by the lithium aluminum hydride reduction of ethylacetoferulate and α,β-diacetoxyacetylhydroferulic acid methylester after Allen and Byers (*13*), and Adler (*14*), respectively. Vanillic acid and syringic acid were prepared by the method of Pearl (*15*). Syringic aldehyde was obtained by the alkaline nitrobenzene oxidation of lignin of beech wood (*16*), and syringoyl methyl ketone was prepared also by the ethanolysis of the lignin of bamboo (*17*).

Propylguaiacol and α,β-diacetoxyacetylhydroferulic acid methylester were kindly provided by Dr. H. Mikawa of the Research Laboratory of the Kokusaku Pulp Industrial Co. Ltd. Dihydroconiferyl alcohol and 4-ethyl guaiacol were provid.d kindly by Dr. H. Matsubara of this Faculty and Dr. T. Yokotsuka of the Noda Industrial Science Laboratory, respectively. Other reagents used were commercial samples.

Manometric Procedure—The following system was used in the Warburg experiments: Main compartment; 20μM of substrates dissolved in 1 ml. phosphate buffer (1/15 *M*, pH 6.8) and distilled water 1 ml. Side arm; Enzyme solutions 0.3 ml. Center well; 15 per cent KOH 0·2 ml. Total volume of fluid 3.0 ml.

The experiments were run at 30°. The influence of CO on the enzyme activity was tested with respirometers filled with a gas mixture of 90 per cent CO+10 per cent O_2, and with that of 90 per cent N_2+10 per cent O_2 as control.

RESULTS

Mushroom (*Psalliota campestris*) *Phenol Oxidase*—The oxygen consumption by *p*-hydroxyphenyl-, 3,4-dihydroxyphenyl-, guaiacyl-, 3,4,5-trihydroxyphenyl- and syringyl derivatives in the presence of mushroom phenol oxidase is shown in Fig. 1, A-D. It can be noted that monophenols inclusive of *p*-cresol and tyrosine were readily oxidized by this enzyme to dark brown products, and the oxidation was characterized by the presence of induction periods of 10–30 minutes (Fig. 1, A, tyrosine) which were removed by the addition of a small amount of catechol. On the other hand, 3,4-dihydroxyphenyl derivatives were rapidly oxidized to melanin like compounds by the enzyme with no induction period (Fig. 1, B). These facts are in agreement with commonly accepted properties of the tyrosinase.

However, the enzyme had a strong oxidizing activity on guaiacyl- and syringyl derivatives as well as on the above mentioned mono- and ortho phenols; nevertheless, the oxidation of these compounds could not be catalyzed by potato tyrosinase as indicated in the following section. Fig. 1, C and D, show the rate of oxygen consumption as a result of enzyme action on guaiacyl- and syringyl derivatives. Of guaiacyl derivatives, dihy-

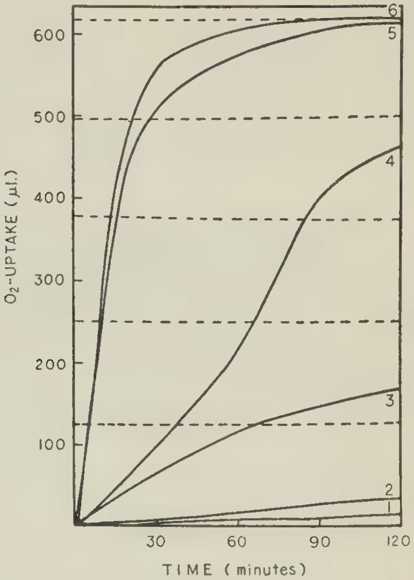


FIG. 1. A. *p*-Hydroxyphenyl derivatives.

1. *p*-hyrdoxybenzoic acid, 2. *p*-hydroxybenzaldehyde, 3. *p*-hydroxycinnamic acid, 4. tryosine, 5. *p*-hydroxyphenylpropionic acid, 6. *p*-cresol.

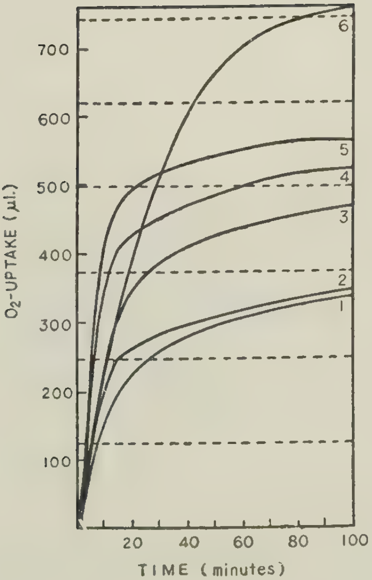
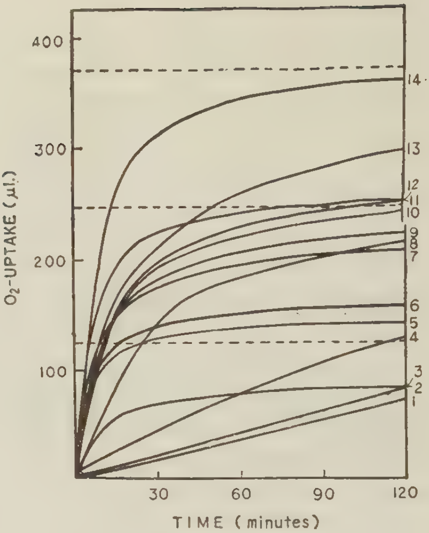


FIG. 1. B. 3,4-Dihydroxyphenyl derivatives.

1. protocatechuic acid, 2. hydroquinone, 3. caffeic acid, 4. protocatechuic aldehyde, 5. catechol, 6. 3,4-dihydroxyphenyl alanine.

FIG. 1. C. Guaiacyl derivatives.

1. vanillin 2. α-hydroxypropiovanillone, 3. isoeugenol, 4. vanilloyl methyl ketone, 5. eugenol, 6. coniferyl alcohol, 7. propylguaiacol, 8. vanillic acid, 9. guaiacyl acetone, 10. ferulic acid, 11 guaiacyl glycerol, 12. 4-ethyl guaiacol, 13. guaiacol, 14. dihydroconiferyl alcohol.



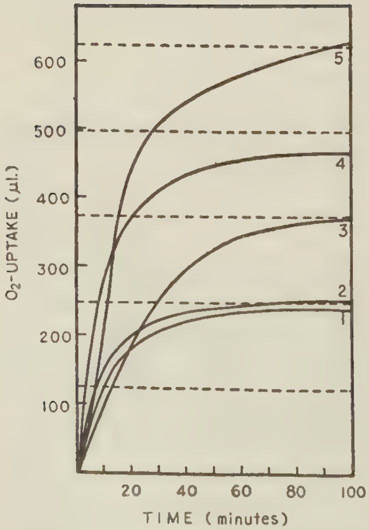


FIG. 1. D. 3,4,5-Trihydroxyphenyl- and syringly derivatives.

1. syringic acid, 2. syringic aldehyde, 3. synap acid, 4. pyrogallol, 5. gallic acid.

The dotted lines indicate successively from the bottom, amounts of oxygen equivalent to 0.5, 1.5, 2.5 and 3.0 atoms per molecule of substrates.

FIG. 1. Oxygen consumption by various substrates in the presence of mushroom phenol oxidase.

droconiferyl alcohol, 4-ethyl guaiacol, propylguaiacol, guaiacylacetone, eugenol, coniferyl alcohol and ferulic acid were oxidized rapidly, while guaiacol, guaiacylglycerol, isoeugenol and vanillic acid absorbed oxygen moderately, and vanilloyl methyl ketone, α-hydroxypropiovanillone and vanillin took up oxygen at slower rates. Further, it is shown clearly in Fig. 1, C that oxygen consumption by coniferyl alcohol as well as by

TABLE I

Color Changes Observed When Mushroom Phenol Oxidase, Laccase of Japanese Lacqueur and Potato Tyrosinase Act on Phenolic Substances

Substrate	Mushroom phenol oxidase	Laccase of Japanese lacqueur	Potato tyrosinase
p-Hydroxybenzaldehyde	None	None	None
p-Hydroxybenzoic acid	None	None	None
p-Cresol	Dark brown	Milky	Dark brown
p-Hydroxyphenylpropionic acid	Brown	Light brown	Brown
p-Hydroxycinnamic acid	Light brown	Light brown	Brown
Tyrosine	Black	None	Black
Catechol	Dark brown	Dark brown	Dark brown
Protocatechuic aldehyde	Dark brown	Dark brown	Dark brown
Protocatechuic acid	Dark brown	Dark brown	Dark brown
Caffeic acid	Dark brown	Dark brown	Dark brown
DOPA	Black	Black	Black
Hydroquinone	Red brown	Red brown	Light red brown
Guaiacol	Red brown	Red brown	Light red brown
Vanillin	Light brown	Light brown	None
Vanillic acid	Light brown	Light brown	None
4-Ethyl guaiacol	Milky	Milky	None
Ferulic acid	Light brown	Light brown	None
Eugenol	Milky	Milky	None
Isoeugenol	Milky	Milky	None
Propylguaiacol	Milky	Milky	None
Coniferyl alcohol	Milky	Milky	None
Dihydroconiferyl alcohol	Milky	Milky	None
α-Hydroxypropiovanillone	None	None	None
Guaiacylacetone	Milky	Milky	None
Vanilloyl methyl ketone	Yellow brown	Yellow brown	None
Guaiacylglycecol	Brown	Brown	None
Pyrogallol	Dark brown	Dark brown	Dark brown
Gallic acid	Dark brown	Dark brown	Light brown
Syringic aldehyde	Red brown	Red brown	None
Syringic acid	Red brown	Red brown	None
Synap acid	Red brown	Red brown	None

eugenol in this case is equivalent to about 0.5 atom per molecule, and the oxidation product of coniferyl alcohol is a pale brownish white polymer as pointed out by Freudenberg (*1*).

Pyrogallol, gallic acid, syringic acid and syringic aldehyde were oxidized rapidly, whereas synap acid was oxidized at a less rapid rate (Fig. 1, D).

Furthermore, a more important fact is that the use of hydroquinone as substrate, for the sake of comparison with the action of the laccase caused a rapid uptake of oxygen (Fig. 1, B).

Table I summarizes the color changes of substrates brought about by the action of mushroom phenol oxidase, laccase of Japanese lacqueur and potato tyrosinase. As seen in Table I, mono- and ortho phenols are oxidized generally by potato tyrosinase to a brown black colored product with a quinone structure. On the other hand, laccase of Japanese lacqueur catalyzes the oxidation of ortho phenols in a similar way, but the effect on monophenols such as *p*-cresol and tyrosine is distinctly different from that of tyrosinase, *viz.*, *p*-cresol is oxidized to a milky product and tyrosine is not oxidized at all. Further, the oxidations of guaiacyl- and syringyl derivatives are catalyzed by laccase but not by tyrosinase. The oxidation products of guaiacyl compounds in many cases are a milky cloudy precipitate.

According to Freudenberg and Richtzenhain (*6*, *18*, *19*), these products may be formed partly by reactions involving the loss of phenolic hydroxyl groups and the formation of ether linkages, *i.e.*, reactions quite similar to those which are assumed to occur in lignin formation.

On the other hand, the oxidation products of syringyl compounds are reddish brown colored polymers which may be considered to possess *o*-quinone structures (*20*).

As for the mushroom enzyme, it may be appropriate to consider judging from the color of oxidation products that the enzyme is a mixture of both laccase and tyrosinase because both tyrosine and *p*-cresol are oxidized to brownish black products (tyrosinase activity), whereas guaiacyl- and syringyl compounds are oxidized respectively to a milky and a red brown product (laccase activity).

If the enzyme is a mixture of both tyrosinase and laccase, and the oxidation of guaiacyl- and syringyl derivatives is catalyzed by laccase, then carbon monoxide should be expected to inhibit the action with monophenols and ortho phenols, as substrates but not with hydroquinone, guaiacyl- and syringyl derivatives, since tyrosinase is without action on the latter substances. Actually, as shown in Table II, carbon monoxide inhibited the oxidation of monophenols, 3,4-dihydroxyphenyl- and 3,4,5-trihydroxyphenyl compounds, whereas the oxidation of hydroquinone, guaiacyl- and syringyl derivatives were not affected. Further, the inhibition of the oxidation of tyrosine and *p*-cresol amounted to 65–70 per cent, while in the case of catechol and pyrogallol, the extent of carbon monoxide inhibition remained only about 40–50 per cent.

Laccase of Japanese Lacqueur--In order to confirm the above mentioned

TABLE II

Influence of Carbon Monoxide on the Mushroom Phenol Oxidase Activity

Substrate	$\mu l.$ O_2 uptake per 10 min. 90% N_2+10% O_2	$\mu l.$ O_2 uptake per 10 min. 90% CO+10% O_2	Degree of inhibition %
p-Cresol	128	44	66
p-Hydroxyphenylpropionic acid	140	65	54
Tyrosine	51*	15*	71
Catechol	175	90	49
Protocatechuic aldehyde	120	85	41
Protocatechuic acid	98	55	44
Caffeic acid	86	52	40
DOPA	94	58	38
Hydroquinone	146	148	0
Guaiacol	68	72	0
Vanillin	15*	14	0
Vanillic acid	30	32	0
4-Ethyl guaiacol	95	96	0
Ferulic acid	60	61	0
Eugenol	80	85	0
Isoeugenol	21	24	0
Propylguaiacol	79	79	0
Coniferyl alcohol	70	72	0
Dihydroconiferyl alcohol	115	112	0
α-Hydroxypropiovanillone	18*	16*	0
Guaiacylacetone	74	76	0
Pyrogallol	180	106	41
Gallic acid	124	80	35
Syringic aldehyde	85	81	0
Syringic acid	79	80	0
Synap acid	72	72	0

* $\mu l.$ O_2 uptake per 30 minutes was read because in the oxidation of tyrosine, 20 minutes of induction period was given, whereas with vanilin and α-hydroxypropiovanillone, the rates of oxidation were remarkably slow.

finding, a similar experiment was made using the well known laccase of Japanese lacqueur. As shown in Fig. 2, B-D, the rates and degrees of oxygen consumption by the enzyme action upon 3,4-dihydroxyphenyl-, guaiacyl- and syringyl compounds are generally in agreement with the results of the preceeding section. The oxygen consumption by both coniferyl alcohol and eugenol were equivalent to about 0.5 atom per molecule, as was the case in the presence of the mushroom phenol oxidase. Further, the oxidation product of coniferyl alcohol in this case was very similar to that of Freudenberg's "Dehydrierungs polymerisate" of coniferyl alcohol. On the other hand, *p*-cresol was oxidized weakly to a pale brownish milky product, whereas tyrosine was not oxidized at all, and the oxidation of *p*-hydroxyphenylpropionic acid was very weak as compared to that by the mushroom enzyme (Fig. 2, A). Above all, it is worthy of note that the oxidation of all the compounds used in the experiment was not inhibited by carbon monoxide. Thus, it is quite probable that the mushroom enzyme is a mixture of both tyrosinase and laccase, and Freudenberg's observation on the oxidation of guaiacyl- and syringyl derivatives by mushroom enzyme should be ascribed to the action of laccase rather than that of a

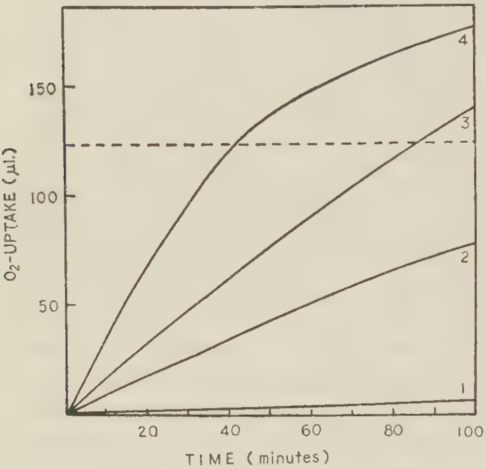


FIG. 2. A. *p*-Hydroxyphenyl derivatives.

1. *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and tyrosine, 2. *p*-hydroxyphenylpropionic acid, 3. *p*-cresol, 4. *p*-hydroxycinnamic acid.

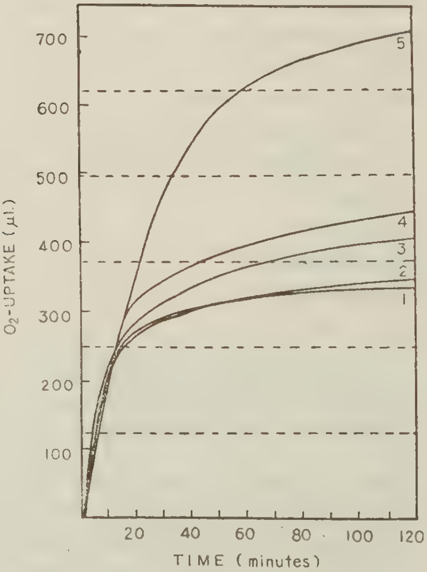


FIG. 2. B. 3,4-Dihydroxyphenyl derivatives.

1. protocatechuic acid, 2. catechol 3. caffeic acid, 4. protocatechuic aldehyde, 5. 3,4-dihydroxyphenylalanine.

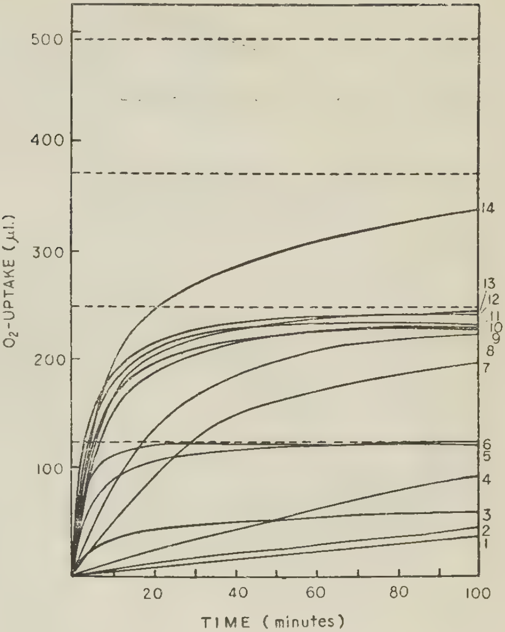


FIG. 2. C. Guaiacyl derivatives.

1. vanillin, 2. α-hydroxypropiovanillone, 3. isoeugenol, 4. vanilloyl methyl ketone, 5. eugenol, 6. coniferyl alcohol, 7. vanillic acid, 8. guaiacylglycerol, 9. ferulic acid, 10. guaiacol, 11. propylguaiacol, 12. guaiacyl acetone, 13. 4-ethyl guaiacol, 14. dihydroconiferyl alcohol.

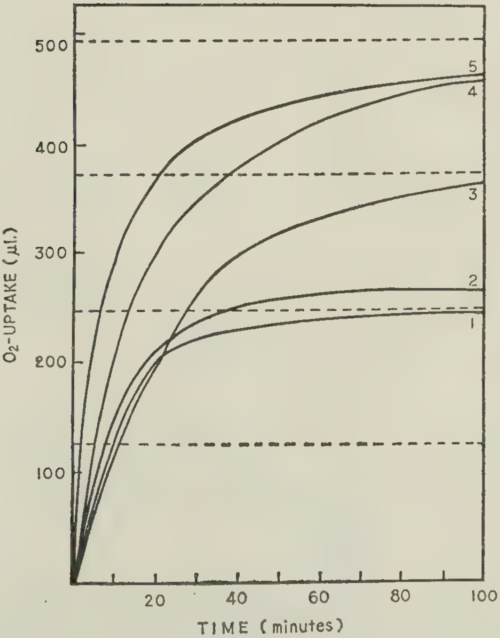


FIG. 2. D. 3,4,5-Trihydroxyphenyl- and syringyl derivatives.

1. syringic acid, 2. syringic aldehyde, 3. synap acid, 4. gallic acid, 5. pyrogallol.

FIG. 2. Oxygen consumption by various substrates in the presence of laccase of Japanese lacqueur.

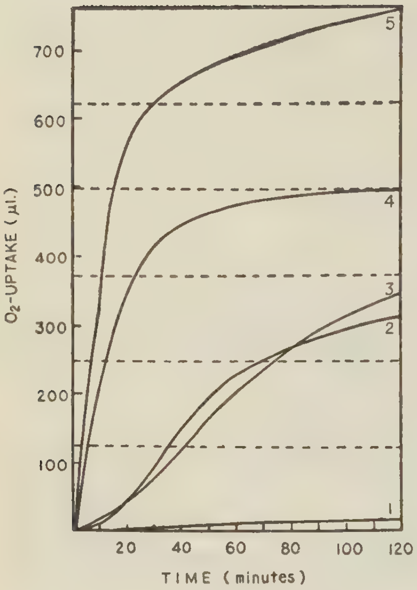


FIG. 3. A. *p*-Hydroxyphenyl derivatives.

1. *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde, 2. *p*-hydroxycinnamic acid, 3. tyrosine, 4. *p*-hydroxyphenylpropionic acid, 5. *p*-cresol.

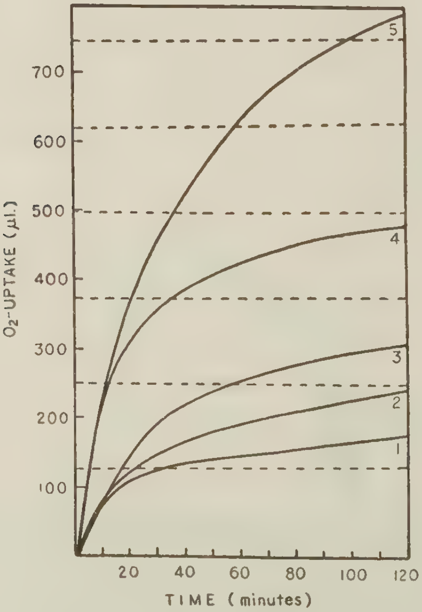


FIG. 3. B. 3,4-Dihydroxyphenyl derivatives.

1. protocatechuic acid, 2. protocatechuic aldehyde, 3. caffeic acid, 4. catechol, 5. 3,4-dihydroxyphenylalanine.

special new enzyme, phenoldehydrogenase.

Potato Tyrosinase—Monophenols and 3,4-dihydroxyphenyl compounds were readily oxidized at a similar rate as observed in the case of the mushroom enzyme (Fig. 3, A and B). Pyrogallol was oxidized rapidly, whereas gallic acid was oxidized at a slower rate.

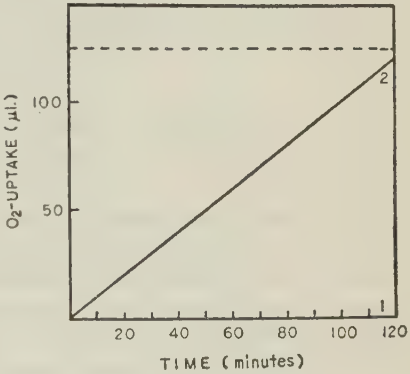


FIG. 3. C. Guaiacyl derivatives.
1. vanillin, vanillic acid, 4-ethyl guaiacol, propylguaiacol, eugenol, isoeugenol, coniferyl alcohol, dihydroconiferyl alcohol, ferulic acid, α-hydroxypropiovanillone, guaiacyl acetone, vanilloyl methyl ketone, and guaiacyl glycerol 2. guaiacol.

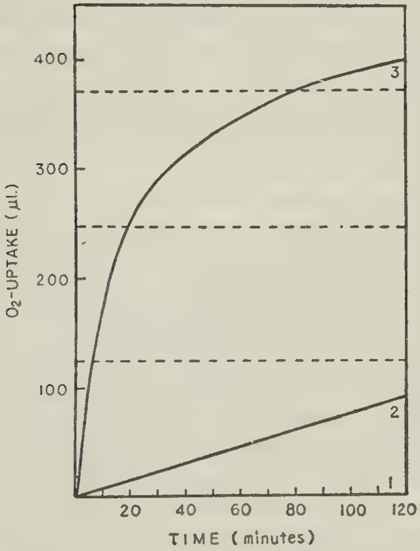


FIG. 3. D. 3,4,5-Trihydroxyphenyl- and syringy derivatives.
1. syringic aldehyde, synap acid, and syringoyl methyl ketone, 2. gallic acid, 3. pyrogallol.

FIG. 3. Oxygen consumption by various substrates in the presence of potato tyrosinase.

However, a striking contrast was found between potato tyrosinase and the above mentioned two enzymes in the oxidation of guaiacyl- and syringyl compounds; of the guaiacyl- and syringyl compounds used in the experiment only guaiacol was oxidized at a slower rate, and the other compounds were not oxidized at all (Fig. 3, C and D, Table I).

Thus, one must conclude that the enzyme obtained from mushroom after Freudenberg's procedure is a mixture of both laccase and tyrosinase, and that the oxidation of monophenols is catalyzed by tyrosinase and that of ortho phenols is catalyzed by both tyrosinase and laccase. On the other hand, the oxidation of guaiacyl- and syringyl derivatives is catalyzed only by laccase, in other words, we have no difficulty in considering that Freudenberg's phenoldehydrogenase is nothing other than a laccase.

DISCUSSION

From our experimental results, it became clear that the enzyme acting on coniferyl alcohol to produce coniferons lignin was actually laccase. Freudenberg (*21*), presumed the existence of laccase (phenoldehydrogenase), the same enzyme as that found in mushroom, in the cambial cells of conifers, since the formation of brown pigment was found to be caused by the cells in the presence of dihydroxyphenylalanine (DOPA). However, the oxidation of DOPA is known to be catalyzed by both tyrosinase and laccase (Fig. 2, B and 3, B), and the distribution of laccase in higher plants is considered to be limited and the laccase present in the latex of the lacqueur trees is rather a exceptional case. Thus, although the oxidation of DOPA found by Freudenberg may be able to explain by the action of laccase, it would be more reasonably explained by the action of tyrosinase which is distributed widely in higher plants.

On the other hand, lignin is known to be a main constituent of the cell walls of higher plants, and also coniferin, a glycoside of coniferyl alcohol, in the cambial sap of conifers has been found.

Freudenberg *et al.* (*21*) demonstrated that the crude enzyme obtained from young shoots of Araucaria excelsa could effect the conversion of coniferyl alcohol into a dehydrogenation polymer, but the yield of the polymer could be increased several folds by the addition of hydrogen peroxide. Theorell (*22*) described that in the presence of hydrogen peroxide, peroxidase oxidizes the same types of compounds as does laccase, and recently Siegel (*23*) found that phenylpropanes such as eugenol were converted into insoluble lignin-like products when incubated with slices of embryonic hypocotyl from the Red Kidney bean in the presence of hydrogen peroxide.

In previous papers, the present author (*24*) reported that the oxidation of coniferyl aldehyde is catalyzed by the enzyme obtained from bamboo-shoot in the presence of hydrogen peroxide, that the localization of peroxidase is confined largely to vascular tissues of bamboo-shoot in which the lignin is being actively deposited, and that the peroxidase attached to the cell wall is fairly stable to heat and is very active. The same observation has also been made by Siegel recently (*25*).

Considering these facts, it may be concluded that the enzymes which are responsible for lignin biosynthesis from coniferyl alcohol are most probably both laccase and peroxidase, and in view of the fact that the peroxidase but not laccase is widely distributed in the higher plants, it may also be inferred that peroxidase plays a main role in lignin biosynthesis as has already been proposed in author's previous papers.

Moreover, it has been confirmed that the dehydrogenation polymers of coniferyl alcohol obtained by the catalysis of both laccase of Japanese lacqueur and radish peroxidase have a chemical nature very similar to that of coniferous lignin, and that ethanolysis and hydrolysis of both polymers lead to the well known Hibbert's ethanolysis monomers*. Analytical results of these polymers in comparison with those of coniferous lignin will be reported in detail elsewhere.

SUMMARY

The phenol oxidase participating in the biosynthesis of lignin has been studied further, and the following facts have been confirmed.

1. The mushroom phenol oxidase preparation obtained by the method of Freudenberg contains a mixture of tyrosinase and laccase, and the oxidation of guaiacyl- and syringyl compounds is catalyzed by laccase.

2. Freudenberg's phenoldehydrogenase which is responsible for the oxidative condensation of coniferyl alcohol can be regarded laccase and not a special new enzyme.

The author wishes to express his sincere thanks to Prof. Dr. I. Kawamura for his kind advice throughout this study. Thanks are also due to Prof. Dr. T. Mori of the Biological Institute, Faculty of Science, Nagoya University for his encouragements during the course of the study.

REFERENCES

(1) Freudencberg, K., *Holz als Roh- und Werkstoff*, **10**, 339 (1952)
(2) Dawson, C. R., and Tarpley, W. B., Copper Oxidases in The Enzyme, Ed. by Sumner and Myrbäck, **II 1**, 489 (1951)
(3) Mason, H. S., and Cronyn, M., *J. Am. Chem. Soc.*, **77**, 491 (1955)
(4) Higuchi, T., Kawamura, I., and Morimoto, I., *J. Jap. Forest. Soc.*, **37**, 446 (1955)
(5) Higuchi, T., *Physiol. Plant.*, **10**, 364 (1957)
(6) Freudenberg, K., and Richtzenhain, H., *Ber. dtsch. chem. Ges.*, **76**, 997 (1943)
(7) Yakushiji, E., *Acta Phytochim.*, **12**, 226 (1941)
(8) Kubowitz, F., *Biochem. Z.*, **292**, 221 (1937)
(9) Cramer, A. B., and Hibbert, H., *J. Am. Chem. Soc.*, **61**, 2204 (1939)
(10) Kulka, M., and Hibbert, H., *J. Am. Chem. Soc.*, **65**, 1180 (1943)
(11) Brickman, L., Hawkins, W. L., and Hibbert, H., *J. Am. Chem. Soc.* **62**, 2149 (1940)
(12) Kratzl, K., and Billek, G., *Monatsh. f. Chemie* **83**, 406 (1953)
(13) Allen, C., and Byers, J., *J. Am. Chem. Soc.*, **71**, 2683 (1949)
(14) Adler, E., and Yllner, S., *Acta Chem. Scand.*, **7**, 570 (1953)
(15) Pearl, I., A., *Organic synthesis*, **30**, 101 (1950)
(16) Creighton, R. H. J., McCarthy, J. L., and Hibbert, H., *J. Am. Chem. Soc.*, **63**, 3049 (1941)

---

* Higuchi, T., unpublished date.

(*17*) Higuchi, T., Kawamura, I., and Ito, Y., *J. Jap. Wood Reseach Soc.*, **2**, 225 (1956)
(*18*) Richtzenhain, H., *Chem. Ber.*, **81**, 260 (1948)
(*19*) Richtzenhain, H., *Chem. Ber.*, **82**, 447 (1949)
(*20*) Freudenberg, K., and Hübner, H. H., *Chem. Ber.*, **85**, 1181 (1952)
(*21*) Freudenberg, K., Reznik, H., Boesenberg, H., and Rasenack, D., *Chem. Ber.*, **85**, 641 (1952)
(*22*) Theorell, H., The Iron-Containing Enzymes. (B. Catalases and Peroxidases), The Enzymes Ed. by Sumner and Myrbäck, II, 1, 397 (1951)
(*23*) Siegel, S. M., *Physiol. Plant.*, **6**, 134 (1953)
(*24*) Higuchi, T., *Physiol. Plant.*, **10**, 621 (1957)
(*25*) Siegel, S. M., *Quart. Rev. Biol.*, **31**, 1 (1956)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

STUDIES ON PROTAMINES

V. CHANGES OF THE PROTEINS IN THE CELL NUCLEI OF THE TESTIS DURING THE FORMATION OF SPERMATOZOA OF THE RAINBOW-TROUT (SALMO IRIDEUS)*

BY TOSHIO ANDO AND CHIEKO HASHIMOTO

(*From the Institute of Science and Technology,*
University of Tokyo, Tokyo)

(Received for publication, March 14, 1958)

In a breeding season of the adult rainbow-trout (*Salmo irideus*), the protamine (*iridine*) prepared from its isolated sperm nuclei has been found to hold molecules, each of which consists of one simple polypeptide chain. This protamine has a simple amino acid composition, containing arginine (90 per cent), serine, glycine, valine, alanine, isoleucine and proline, and a molecular weight of *ca.* 5,000. (*1*)

Chemical studies on the protamine (*cyprinine*) of carp testis connected with the biosynthesis of protamine were once attempted by Kossel *et al.* (*2*). They found that there were a kind of histone (cyprinohistone) and some basic peptones together with cyprinine in the immature testis of a carp.

In the present case of the rainbow-trout, it is a way of approach to the course of biosynthesis of the protamine in the testis, to learn when an accomplished protamine (*iridine*) starts to be formed in the testis cell nuclei. It is also necessary for that purpose to study the relation of the protamine to nucleic acids and other basic proteins found together therein during the period of spermatozoa formation, and to compare the chemical structure of the basic proteins with one another on the basis of results to be obtained by chemical and enzymic analyses.

In the present paper, the results are described, which have been obtained from such analytical studies on sperm and testis cell nuclei of the rainbow-trout collected every month for one year. The results have been confirmed on the materials collected through three years.

EXPERIMENTAL

a) *Material*—As described in the previous papers, testes of rainbow-trouts (*Salmo irideus*) were obtained from the fish hatchery of Tokyo city at Okutama, Tokyo**. The testes were excised from a few trouts of 4 to 5 years old collected at the beginning of every month for a year. The material was transported to our laboratory in a container filled with natural or dry ice, within three hours after collection. All the treatments described below for the isolation of nuclei and the preparation of basic proteins were made at 0 to 4°.

---

* Presented at the International Symposium on Enzyme Chemistry (Tokyo, October, 1957) and partly at the 28th and 29th Annual Meetings of the Japanese Biochemical Society (Tokyo, November, 1955 and Fukuoka, October, 1956).

** We are much indebted to Mr. S. Ohuchi, the superintendent of the Okutama branch of the Tokyo City Fisheries Experimental Station for the material.

b) Isolation of Testis Cell Nuclei—This was carried out by the method described in the previous paper (*1*), using Ringer's solution for fresh-water fish. This treatment was done with the utmost care, for the cell nuclei in the immature testes were very unstable. Following up the above process of isolation with photo- and electronmicroscopic observations (*3*), it was seen that the sperm and cell nuclei always remained without any apparent deformation after initial centrifugation (see Fig. 3).

c) Preparation of Basic Proteins from the Isolated Nuclei—By extracting the isolated nuclei, prepared each month, with 0.2 *N* hydrochloric acid, the basic proteins were obtained and precipitated as the picrates. They were fractionated by making use of the solubility difference in 67 per cent aqueous acetone and then converted to their sulfates. After repeated purification with water and alcohol, the basic protein sulfates ware obtained as glittering powder.

d) Composition Analysis of the Isolated Nuclei and Basic Proteins—The phosphorus content was determined by the methods of King (*4*) and Schmidt-Thannhauser (*5*), and arginine content by the method of Sakaguchi (*6*). The amino acid composition of each specimen of the isolated nuclei and purified basic protein sulfates was investigated qualitatively and semi-quantitatively. For the identification of the component amino acids, 10 mg. each of specimens was hydrolyzed with 20 per cent hydrochloric acid at 110° in sealed tube for 32 hours, and the product was subjected to a two-dimensional paper chromatography using phenol-ammonia and butanol-formic acid systems as solvents.

e) Fractionation of the Basic Proteins—The basic protein sulfates obtained from the testis nuclei in October were fractionated into soluble and insoluble parts by applying twice the isoelectric precipitation method at pH *ca.* 10.5 for histone. Each part was further fractionated by dialysis using parchment paper to yield two parts. Thus, four fractions were tentatively obtained. On the other hand, 2,4-dinitrophenyl (DNP)-derivatives of the basic protein sulsates from the testis nuclei were prepared, and were fractionated to yield two parts by utilizing their solubility difference in water. The water-soluble fraction was purified by using a column of activated alumina (13 g., 1 × 15 cm.) (*7*) or columns of Amberlite XE-64 (H-form) and then IR-4B. The DNP-derivative retained in the column, was eluted with 0.1 *N* hydrochloric acid and then transformed into the sulfate. From the less soluble fraction, the sulfate was also obtained.

It was found possible by the latter method (the fractionation of the DNP-proteins) to separate tentatively the DNP-basic protein of the protamine (iridine) type from the DNP-derivatives of the other types of basic proteins (non-iridine type). Accordingly, the DNP-derivatives of the basic proteins obtained in September, November and December were fractionated, respectively, into the DNP-proteins of iridine type and of non-iridine type by employing the latter method.

f) Structure Analysis of the Basic Proteins in the Testis Nuclei and Some Fractions Obtained from Them—The DNP-derivatives of the unfractionated basic proteins of sperm- and testis cell-nuclei obtained in various months (September-March) and the fractionated DNP-proteins of the iridine type and non-iridine type were hydrolyzed respectively by heating in sealed tube at 105° for 16 hours with 20 per cent hydrochloric acid or 10 hours with 35 per cent hydrochloric acid. DNP-amino acids yielded were identified and determined by means of two-dimensional paper chromatography according to Levy (*8*). Furthermore, the amino acids were cofirmed by regeneration through ammoniacal decomposition of the eluates of the spots on the paper.

On the other hand, the absorption spectrum of a purified DNP-basic protein of iridine type was determined, and from the result the N-terminal residue of the basic protein was identified, and its molecular weight was estimated according to the method previously reported (*7*).

In order to compare the C-terminal residues of these basic proteins, carboxypeptidase* and an enzyme fraction** containing basic carboxypeptidase besides, as described in the previous paper (*1*), were used for the unfractionated basic proteins in October and November. The enzymes were treated with DFP (diisopropyl fluorophosphate) just prior to the use.

Peptic digestion of iridine obtained in March and the basic proteins during September to December, was made at pH 2.1 in a 0.02 per cent pepsin (Sigma Chem. Co.) solution, respectively. The products were investigated for the increase in amino groups by ninhydrin method and for the N-terminal residues by DNP-method.

g) *Isolation and Identification of the Amino Acids and the Basic Peptides Contained in Testis*—The testes obtained in August-March, were extracted with 60–80 per cent alcohol, respectively. After being dried, the extract was desalted. The amino acids thus obtained were identified by a two-dimensional paper chromatographic methd.

From the supernatants obtained, when the sperm- and the testis cell-nuclei were isolated, the basic peptides were precipitated as phosphotungstic acid salts, from which the peptides were obtained according to the method of Felix *et al.* (*9*).

RESULTS AND DISCUSSION

Average weight of each fresh testis obtained from 4 year old rainbow-trouts during June-August (forming fibrous tissue) was *ca.* 1 g. and it increased rapidly to *ca.* 15 g. from August to October (forming completed testis). No more increase was found in the weight of testis after October, until the

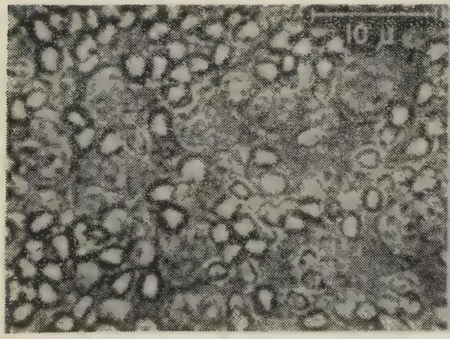


FIG. 1. A photomicrograph of spermatozoa and germ cells in an immature testis (October) of a rainbow-trout 4 years old, stained with acetocarmine.

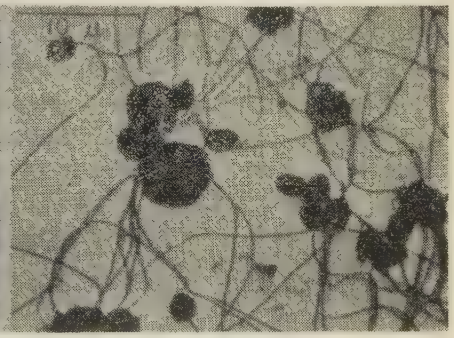


FIG. 2. An electronmicrograph of spermatozoa and germ cells in an immature testis (October) of a rainbow-trout 4 years old.

---

* Prepared and made to act upon the basic proteins by Dr. Tobita in our laboratory.

** Obtained by Dr. Tobita in this laboratory in the course of the preparation of carboxypeptidase and shown to contain basic carboxypeptidase besides, using carbobenzoxyglycyl-L-arginine as substrate.

breeding season is over. The conspicuous morphological features of spermatozoa and germ cells in an immature testis (during a spermatozoon-forming season, June-December), were observed by means of a photo- and an electron-microscope*. The typical micrographs are shown in Figs. 2 and 3. Few germ cells were found in testes obtained in June, July and August, but it was seen that they multiplied rapidly in each testis in September. Besides germ cells, spermatozoa were found to be already formed in October.

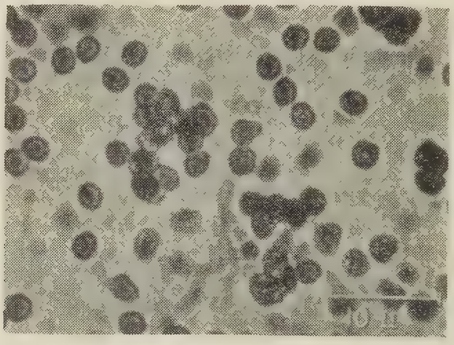


FIG. 3. A photomicrograph of nuclei isolated from an immature testis of a rainbow-trout 4 years old obtained in November, stained with acetocarmine.

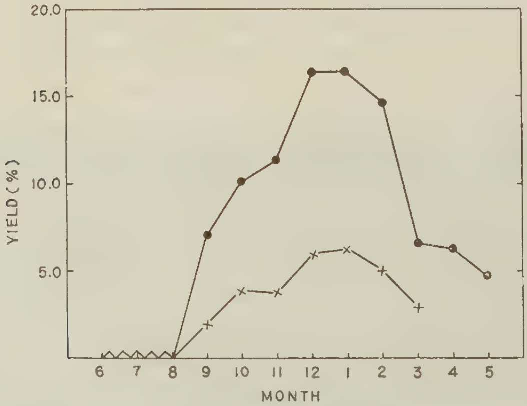


FIG. 4. Yield (%) of isolated nuclei (●) and of basic protein sulfates (×) per one fresh testis.

While very little formation of new germ cells was found in November, the formation of spermatozoa from spermatids was observed. Only spermatozoa were found in a testis obtained in December, when the spermatogenesis was presumably completed.

---

* We are indebted to Mr. S. Sakata in the laboratory of Electron Microscopy, Tokyo University for the photo- and electronmicroscopic techniques.

TABLE I

Composition of Isolated Testis Nuclei[1]

	N[2]	P[3]	N/P	PNA-P[3]	Arg[4]	P : Arg	Yield of basic proteins	Residue of acid extraction
	%	%		%	%		%	%
Sept.	16.4	3.3	5.0	0.2_4	$12._1$	1 : 0.7	26	62
Oct.	18.7	4.7	4.1	0.1_1	$25._6$	1 : 1.0	34	66
Nov.	19.6	5.4	3.6	0.0_6	$32._4$	1 : 1.1	33	63
Dec.	18.8	5.2	3.6	0.0_5	$32._5$	1 : 1.1	36	63
Feb.	20.6	5.6	3.6	0.0	$33._0$	1 : 1.0	38	64
Mar.	20.6	5.6	3.6	0.0	$33._1$	1 : 1.0	38–42	—

1) Values are given in percentage of weight of dried isolated nuclei.

2) Obtained by Kjeldahl's method.

3) Obtained by King's method (*4*) and Schmidt and Thannhauser's method (*5*).

4) Obtained by Sakaguchi's method (*6*).

TABLE II

Changes in the Amino Acid Composition[1] *of Isolated Testis Cell Nuclei from 4–5 Year Old Rainbow-Trout during the Formation of Spermatozoa*

	(June)[2]	(Aug.)[2]	Sept.	Oct.	Nov.	Dec.	Jan., Mar.	Apr., May
Total N (%)	—	—	16.4	18.7	19.6	18.8	20.2 20.6	20.2 19.5
Arg	++	+	+	++ ++	++ ++	+++ ++	+++ ++	+++ ++
Leus	+++	+++	+++	+++	+++	++	++	+
Thr	—	—	—	+	+	±	±	—
Lys	++	++	++	+++	++	+	+	+
Glu	++	+++	++	++	++	+	+	++
Asp	++	++	++	++	++	+	+	+
His	?	+	+	+	+	±	—	—
Tyr	—	—	+	+	+	—	—	—
Phe	—	—	±	+	±	—	—	—
(Cys)[3]	+	+	+	+	—	—	—	—

1) The following amino acids were always found besides those shown in this table: Gly, Ser, Val, Ala and Pro, each in an amount to be shown as +++ or ++. Try was absent throughout these months. + or — shows relative color yield by ninhydrin reaction on a two-dimensional paper chromatogram (phenol-0.1 per cent NH_3 and butanol-formic acid system) per hydrolysate of a unit weight of protein.

2) A dried testis was used for analysis, instead of isolated nuclei.

3) The spot of cystine is somewhat uncertain.

As shown in Fig. 4, neither sperm nuclei nor testis nuclei could practically be isolated from the testes obtained in June-August, but it was recognized that these nuclei multiplied rapidly with the enlargement and maturation of a testis in September-November.

The results of the composition analyses of these isolated nuclei are shown in Table I and II. Nitrogen, phosphorus and arginine contents increased gradually, the ratios of N/P decreased from 5.0 to 3.6, and those of P/Arg from 1.5 to 1.0, with the maturation of testis. They gave nearly constant values respectively after November. The presence of phosphorus of pentosenucleic acid (PNA) in addition to that of deoxyribonucleic acid (DNA) was found in isolated nuclei obtained in September and October, but it decreased more and more with the maturation of the testis. Finally, only phosphorus of DNA could be found in the nuclei after November. As for the amino acid composition per unit weight of isolated nuclei in every month of one year, a marked increase was found in the amount of arginine with the testis maturation, whereas some other components, cystine*, phenylalanine, tyrosine, histidine, threonine and lysine descreased and disappeared in that order.

TABLE III

Changes in the Amino Acid Composition1) of Basic Proteins in Isolated Testis Nuclei from 4–5 Year Old Rainbow-Trout during the Formation of Spermatozoa

	Sept.	Oct.	Nov.	Dec. (b)2)	Dec. (a)	Jan.-Mar.
Total N (%)	(15.2)2) 16.8	19.5	20.5	–	22.6	23.0
Arg	(+)2) ++	++ ++	+++ ++	+++ ++	+++ ++	+++ ++
Ala	+++	+++	+++	++	+	+
Leus	+++	+++	++	++	+	+
Thr	+	++	±	–	–	–
Lys	++ ++	+++	++	+	±	–
Glu	++	++	+	+	–	–
Asp	+	++	+	+	–	–
His	+	+	±	–	–	–
Tyr	+	+	–	–	–	–
Phe	+	+	–	–	–	–
(Cys)3)	+	+	+	–	–	–

1) In addition to these amino acids, Gly, Ser, Val and Pro were always present (+++ or ++). Try was absent. + or –: see the footnote of Table II.

2) The basic proteins obtained from the less soluble picrates in 67 per cent aqueous acetone.

3) The spot of cystine is somewhat uncertain.

---

* See the footnote of Table II.

The yield, nitrogen content, and amino acid composition of the basic proteins, obtained from the isolated testis nuclei, are summarized in Fig. 4, Tables I and III, respectively. Quantitative amino acid compositions of the basic proteins prepared from the isolated sperm nuclei in a breeding season were shown in the previous paper (*1*). The amount of the basic proteins per one testis increased with the increase in the yield of isolated testis nuclei and nucleic acid content, as indicated by the phosphorus value.

TABLE IV

Fractionation and Amino Acid Composition[1] *of the Basic Proteins*[2] *in Testis Nuclei Obtained in October*

	DNP-Derivative method		Isoelectric precipitation-dialysis method			
	DNP-Protein of non-iridine type	DNP-Protein of iridine type	Non-protamine type protein (histone-like)		Protamine type protein	
			a	b	c	d
Yield[3] (%)	44.4	49.6	10	10	38	5
Total N (%)	--	--	18.3	20.8	21.5	21.2
Arg (%)	--	81.6	24.2	--	--	84.5
Arg	⧻	⧻ ⧺	⧻	⧺	⧻ ⧺	⧻ ⧺
Ala	⧻	+	⧻	⧻	⧺	⧺
Leus	⧻	+	⧻	⧻	⧺	+
Thr	⧺	−	⧻	⧺	+	?
Lys	+	−	⧻	⧻	⧺	+
Glu	⧺	−	+	+	+	−
Asp	⧺	−	⧺	+	+	−
His	+	−	+	+	+	−
Tyr	+	−	⧺	+	+	−
Phe	+	−	⧺	+	+	−
(Cys)[4]	+	−	+	+	±	−

1) In addition to these amino acids, Gly, Ser, Val and Pro were always present (⧻ or ⧺). Try was absent. + or −: see the footnote of Table II.

2) These materials are the fraction (yield: 51 per cent of the total basic protein) easily soluble in 67 per cent aqueous acetone and separated from the total basic protein picrate, which was prepared from the isolated testis nuclei in October, 1954.

3) Yield of the fraction per basic proteins used.

4) The spot of cystine is somewhat uncertain.

However, these basic proteins were so heterogenious that they were separated into several fractions by the following three methods: (i) The utilization of the solubility difference of their picrates in 67 per cent aqueous acetone, (ii) the precipitation at the isoelectric point of histone followed by dialysis with parchment paper (as shown in Table IV), and (iii) the utilization of the solubility difference of the DNP-derivatives of the basic proteins in water (see Table IV). The basic proteins obtained each month increased from 15 per cent to 23 per cent in nitrogen content with the maturation of spermatozoa formation (Table III). As to the amino acid composition per unit weight of the basic proteins, contrary to the evident increase in arginine with the maturation, phenylalanine, tyrosine, threonine, histidine, cystine, glutamic acid, aspartic acid and lysine decreased and disappeared in that order. Finally, during the breeding season, the basic proteins of the isolated nuclei were found to change into protamine (iridine), which has a simple amino acid composition shown in Table III (January-March). These changes, as indicated above, showed the same tendency as those of isolated nuclei.

From these results, it may be concluded that the basic protein multiplies with the DNA of the nucleoprotein in the cell nuclei of a whole testis from August to October. After that, very little multiplication of DNA in the whole of the testis was apparently recognized, but there was found a rapid increase in the basic protein, protamine, which contains great quantities of arginine as residues capable of combining with the phosphorus residues of DNA.

The four fractions separated from the basic proteins in the testis nuclei obtained in October, by means of precipitation at an isoelectric point of histone and dialysis as shown in Table IV, had some differences in nitrogen and arginine contents, and amino acid compositions, but no satisfactory fractionation was expected. By another method, however, making use of the solubility difference of the DNP-basic proteins in water, DNP-basic protein of iridine type which had the same amino acid composition as iridine itself in a breeding season, could be fractionated from the other DNP-basic protein of non-iridine type. Hence, by the same method, the iridine type and the other type proteins were fractionated as their DNP-derivatives from the basic proteins obtained each month except October. The results obtained are summarized in Table V. It was found that, of these basic proteins, the iridine type protein increased, while the non-iridine type one decreased with the maturation of the formation of spermatozoa. Furthermore, the majority of the basic proteins in December consisted of protein of iridine type, and it had a behavior similar to iridine in January-March. It is considered that a biosynthesis of iridine as revealed by chemical evidences was parallel to the maturation in forming spermatozoa observed by microscopy.

On applying DNP-method it was pointed out, that the N-terminal residues of the unfractionated basic proteins, obtained each month, were alanine and a little proline in September, both alanine and proline in October, proline and a little alanine in November, and only proline after December (see also Table V). Besides, ε-DNP-lysine was identified in the hydrolysates in every period of the time of immaturity. There is only a

TABLE V

Structure Analysis and Fractionation of DNP-Derivatives of the Basic Proteins Obtained in Various Months from Testis Nuclei of Rainbow-Trout.

	As DNP-amino acid		As DNP-basic protein[1]	
	N-Terminal	Other deriv.	Iridine type[2]	Non-protamine type
			%	%
Sept.	Ala, (Pro)[3]	ε-DNP-Lys	0	98 (98)
Oct.	Ala, Pro	ε-DNP-Lys	26 (50)	71 (44)
Nov.	Pro, (Ala)[3]	ε-DNP-Lys	50 (61)	36 (22)
Dec.	Pro	(ε-DNP-Lys)[3]	86 (87)	5 (5)
Jan., Feb.	Pro	—	89	0
Mar.	Pro	—	90	0

[1] Approximate yield of the DNP-derivative of iridine type or other type protein per total basic proteins obtained from isolated sperm and testis nuclei. Parenthesized values show the yield of each DNP-protein per basic proteins used.

[2] All the N-terminal residue of this type protein was found to be proline. See also the description in the text.

[3] Parenthesized amino acid or its derivative was detected only in slight quantity.

little ε-DNP-lysine in December, and none of it was found from January to March. It was also found that all the N-terminal residue of the iridine type protein fractionated in October-March by the above treatment was occupied by proline, while that of the non-iridine type protein, by alanine and proline. From the studies of the absorption spectrum of each DNP-iridine type protein, its N-terminal residue was identified as proline and its molecular weight of *ca.* 5,000 was estimated. It was also found to contain 82–84 per cent of arginine in the DNP-protein.

As for the C-terminal residues of the basic proteins, results have been obtained which support arginine as the greater part of the termini. As shown in the previous paper (*1*), the greater part of the C-terminal residue of iridine, obtained from trouts of 5 and 2 years old in a breeding season, was found to be occupied by arginine by the hydrazinolysis method (release of ornithine) and the digestion with DFP-treated carboxypeptidase (no release). When the basic proteins obtained in October were incubated with DFP-treated carboxypeptidase, they were also found to be entirely resistant to the enzyme*. In order to assure the result, an enzyme fraction, which was prepared from beef pancreas and shown by Dr. Tobita in our laboratory to contain basic carboxypeptidase besides carboxypeptidase, was also used for the digestion. When the basic proteins obtained in November were incubated with the enzyme fraction treated by DFP, it was found that only arginine was released from the proteins at the incubation periods of 2, 8 and 24 hours, as in the case of iridine obtained in March. Detailed description on the nature and the action of the enzyme fraction upon protamines will be fully given later by one of the present authors (T.A.) and Tobita.

---

* The carboxypeptidase digestion experiment was kindly made by Dr. Tobita in our laboratory. Details will be later reported by him elsewhere.

For the purpose of comparing the behavior of the basic proteins towards protease, those obtained during September to December, and in March, were incubated with pepsin for 12 and 24 hours (48 hours also for the protein in March). Iridine in a breeding season and the basic proteins in December were both resistant to the enzyme. In contrast, the basic proteins during September-November were hydrolyzed to some extent, shown by the investigation of the amino groups and the N-terminal residues appeared in the digest and also of the yield of the pepsin resistant core, as summarized in Table VI. These details will be later discussed elsewhere.

TABLE VI

Analyses of Peptic Digestion Products[1] *of the Basic Proteins Obtained in Various Months*

	Peptic digestion product		Yield[2] of resistant core
	Increase in ninhydrin amino groups	N-Terminal residues	
	Mole/10³ g. protein		%
Sept.	1.9_4	Ala > Lys ≫ Asp, Phe > Ser, Leus, Val, Thr, Tyr | Pro	28
Oct.	0.4_5	Lys > Asp, Ser > Phe > Thr > Ala > Leus, Val | Pro	64
Nov.	0.2_2	Ala, Ser | Pro	90
Dec.	—	Pro	—
Mar.	0.0_3	Pro	105

1) The digestion was carried out by incubating with pepsin for 24 hours at 37°.

2) These products were obtained as sulfates.

As for the amino acids contained in a free state in one testis each obtained in August-March, all of the amino acids proved in the non-protamine type protein were found, but the total content was very small (0.07 per cent) in quantity per one fresh testis. It is of interest that the free arginine was especially small in quantity as compared to other amino acids, and that the basic peptides in a free state, containing arginine at least, amounted only to *ca.* 0.01 per cent of one fresh testis and appeared to be very small ones.

From these results, it is inferred, that while the basic protein of testis nuclei in a breeding season has been shown in the preceding paper (*1*) to consist of only iridine, all the iridine type protein, fractionated from the basic proteins obtained from October through December, also has probably the same chemical structure as that of iridine. But it may be considered, that non-iridine type protein is a mixture of a variety of basic proteins containing lysine besides arginine.

For the purpose of approach to the course of biosynthesis of the protamine in the testis cell nuclei, still many studies are needed from various points of view.

SUMMARY

1. In order to obtain information concerning the biosynthesis of nucleoprotein in cell nuclei of a testis during the process of spermatozoa formation of a rainbow-trout, compositions of sperm- and cell-nuclei, isolated from testes obtained in every month of one year, and those and the chemical structures of the basic proteins prepared from these nuclei were studied for three years.

From the results, it was inferred that during the season of rapid multiplication of testis cells (from August to October), both the basic proteins (mostly proteins of non-protamine type) and the DNA seem to be increased in the same period in the whole of testis nuclei. PNA was also found to appear in the same season, but it decreased and disappeared with the maturation of the testis. After that, with the maturation in spermatozoa formation (from October to December), no apparent increase in DNA was seen in testis nuclei. At that time, while the proteins of the non-protamine type in the nuclei decreased and disappeared, the protein of the protamine type which contains an equivalent quantity of arginine to combine with the total phosphorus residues in DNA, was found to increase gradually.

2. Formation of the iridine type protein (protamine) in the testis nuclei as revealed by chemical analyses and enzymic investigations, was found to run parallel to the maturation in spermatozoa formation, which was observed by means of photo- and electronmicroscopes. It was also recognized, that all the protamine type protein fractionated from the basic proteins in the testis nuclei in every month from October to December, has presumably the same chemical structure as that of an accomplished protamine, iridine, obtained during a breeding season.

The authors are indebted to Dr. N. Ui for his valuable suggestion in treatment of histone, and to Drs. M. Yamasaki and T. Tobita in this laboratory for their kind advice in enzymic digestion. The expense of this work was aided in part by the Scientific Research Grant from the Ministry of Education, to whom the authors are grateful.

REFERENCES

(*1*) Ando, T., and Hashimoto, C., *J. Biochem.*, **45**, 453 (1958)
(*2*) Kossel, A., and Schenck, E. G., *Z. physiol. Chem.*, **173**, 278 (1928)
(*3*) Hashimoto, C., and Sakata, S., The 11th Annual Meeting of the Society of

Electron-Microscopy, Japan (Yokohama, May, 1955)
(4) King, E. J., *Biochem. J.*, **26**, 292 (1932)
(5) Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945)
(6) Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950); **38**, 91 (1951)
(7) Hashimoto, C., *Bull. Chem. Soc. Japan*, **28**, 385 (1955)
(8) Levy, A. L., *Nature*, **174**, 126 (1954)
(9) Felix, K., Rauen, H. M., and Zimmer, G. H., *Z. physiol. Chem.*, **291**, 228 (1952); Rauen, H. M., Stamm, W., and Felix, K., *ibid.*, **291**, 275 (1952)

The Journal of Biochemistry, Vol. 45, No. 7, 1958

ACTION SPECTRA OF PHOTOSYNTHESIS AND PHOTOCHEMICAL ASSIMILATION OF PYRUVIC ACID IN RHODOPSEUDOMONAS PALUSTRIS

By KEN TAKAMATSU AND SIGEHIRO MORITA

(*From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo*)

(Received for publication, April 28, 1958)

In a previous report from this laboratory, one of the present authors (Morita, (*1*)) showed that the cells of *Rhodopseudomonas palustris* perform, in the absence of carbon dioxide, a process of photochemical assimilation of pyruvic acid under anaerobic conditions. Experimental evidence has been marshalled for the occurrence of the reaction:

$$CH_3COCOOH + 1/2\,H_2O \xrightarrow[\text{light}]{} 5/2\,(CH_2O) + 1/2\,CO_2$$

which is different from the ordinary process of bacterial photosynthesis, in which various organic substances (including pyruvate) function as hydrogen donors for the reduction of carbon dioxide.

The present work is concerned with the action spectrum of this photochemical process as compared with that of authentic photosynthetic process (using propionic acid as the hydrogen donor) which has been known to involve the action of bacteriochlorophyll as the photo-catalyst (*2*).

METHODS

Bacterial Cells—A strain of *Rhodopseudomonas palustris*, originally isolated and in continual use in this laboratory, served as the test organism. The bacterium was grown in a peptone-lactate medium, containing 0.5 per cent peptone ("Kyokuto") and 0.5 per cent lactic acid. After 72 hours of culture at 27° under continuous illumination (about 3,000 lux), the cells were harvested and washed several times with water.

Optical Equipment—Monochromatic lights were obtained by the use of interference filters in combination with appropriate sets of colored glass filters. The light from a 1 kw incandescent lamp was passed through a 5 cm. layer of water, two sheets of colored glass filter, and, finally, an interference filter, mounted in front of the glass window of a water thermostat (Fig. 1). In each experiment, the position of the incandescent lamp was adjusted so as to give an incident light of 2×10^3 erg/sec. cm^2, a level which had been ascertained, with all wavelengths studied, to be sufficiently low to give a linear proportionality between the light energy and the physiological activities to be investigated. The light intensity was measured at the position of the test vessel (see Fig. 1) with a Kipp thermopile, model E 2, which had been calibrated with a Standard Lamp, C-597, of the U.S. Bureau of Standards. The spectral characteristics of the monochromatic lights obtained with various combinations of the filters are shown in Table I and Fig. 2.

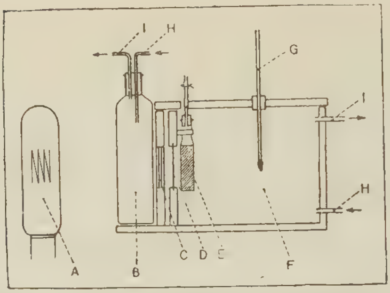


FIG. 1. Assembly of apparatus, side view.
A: incandescent lamp (1 kw)
B: Roux's flask with circulating water
C: filter system
D: glass window of water thermostat
E: test vessel
F: water thermostat
G: thermometer
H and I: inlet and outlet of thomostated water.

TABLE I

Monochromatic Lights Used in the Experiments

Notations used in Fig. 2	Combination of filters			Maximum transmittance at
	Glass filter " Toshiba "	Glass filter " Baird "	Interference filter " Baird "	
A	V-V2-1 V-V2-2	—	—	395 $m\mu$
B	V-B3A-1	B1	V-412-136	455
C	V-Y1A-1	B6	V-462-151	510
D	V-G1A-2	Y2	V-358-122	534
E	V-G1A-1	Y5	V-361-123	552
F	IRO-1B-2	Y3	7-5189-6	597
G	IRO-1B-1	R6	7-5177-1	645
H	IRO-1B-2	R3	V-51	702.5

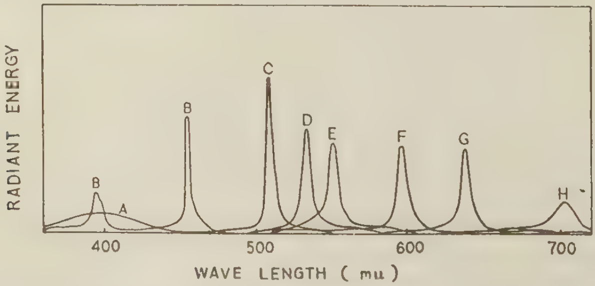


FIG. 2. Monochromatic lights used in the experiments.
The notations A to H correspond to the various combinations of filters listed in Table I.

Measurement of Photochemical Processes—The bacterial suspension was placed in vessel (35 ml. in capacity and 16 mm. in thickness) with a tight-fitting rubber stopper carrying a glass tube for the removal and exchange of gas (see Fig. 1). Usually, 25 to 40 mg. (on a dry weight basis) of washed bacterial cells were used per vessel. The composition of the basal reaction medium was the following: 1 ml. bacterial suspension, 4 ml. $M/5$ phosphate buffer (pH 7.0), and 19 ml. water. In the determinations of pyruvic acid assimilation and photosynthetic reaction, 1 ml. each of $M/400$ sodium pyruvate and $M/5$ sodium propionate solution, respectively, were added to 24 ml. of the basal medium. In both series of experiments, the gas space of the vessel was filled with nitrogen; to secure the strict anaerobic condition, a small piece of white phosphorus was hung with a wire in the gas space under the rubber stopper. All tests were run at 27°.

In the experiment of photochemical assimilation of pyruvate, the reaction was started by introducing, with an injection syringe, 1 ml. of the solution of sodium pyruvate through the rubber stopper, and at the same time the light was turned on. After a reaction period of 60 minutes, the reaction was stopped by injecting 5 ml. of 20 per cent trichloracetic acid, and the amount of pyruvic acid in the reaction mixture was determined by Friedman and Haugen's method modified by Shimizu (*4*).

Photosynthetic activity was measured by determining the amount of $C^{14}O_2$ assimilated in the presence of propionic acid as the hydrogen donor. The reaction was started by injecting—simultaneously with turning on the light—1 ml. of $M/5$ sodium bicarbonate solution (C^{14} labeled) into the test vessel containing the reaction mixture described above. After blocking the reaction with 5 ml. of 20 per cent trichloracetic acid solution, the cells were collected by centrifugation, dried, and the radioactivity of assimilated carbon was measured with a Geiger-Müller counter.

RESULTS AND DISCUSSION

The action spectrum obtained for the process of photochemical assimilation of pyruvate and that for the photosynthetic activity are presented in Figs. 3 and 4. For comparison the absorption spectrum of the living cell suspension was also measured (Fig. 5).

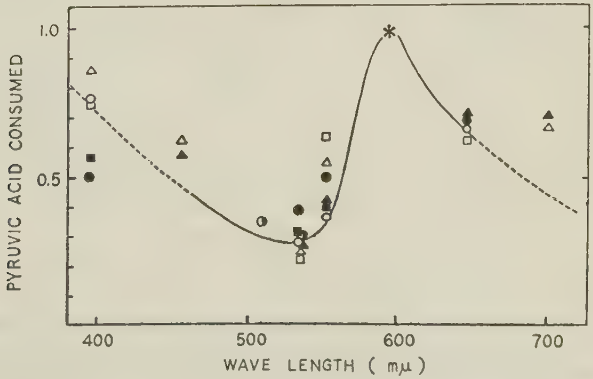


FIG. 3. Action spectrum of photochemical assimilation of pyruvate. For the unit of relative activity, see text.

In Fig. 3, the relative activities at different wavelengths are expressed in percentage of the activity observed at 598 mμ, which is indicated by an asterisk on the curve. The photosynthetic activity in Fig. 4 is presented as the amount of C^{14} assimilated by 1 mg. dry weight of the bacterium in a

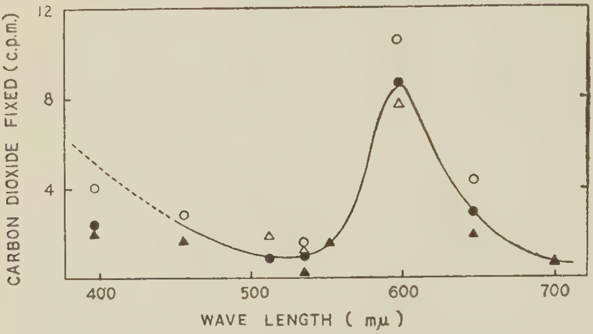


FIG. 4. Action spectrum of photosynthetic CO_2 assimilation in the presence of propionate.

The photosynthetic activity is expressed in terms of radioactivity of incorporated C^{14} per mg. dry weight of bacterial cells in 60 minutes at 27°. Bacterial cell: 25.7 mg. per vessel. Gas space was filled with nitrogen.

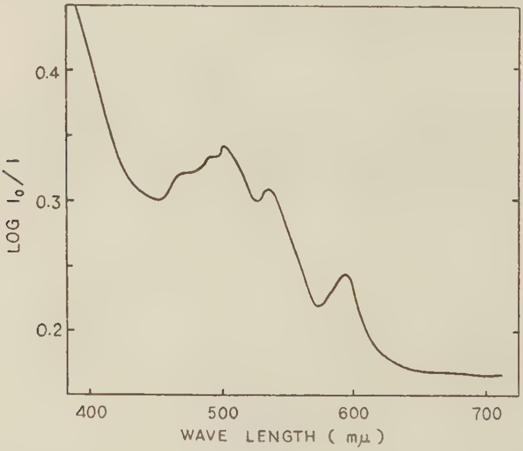


FIG. 5. Absorption spectrum of intact cell of *Rhodopseudomonas palustris* suspended in water.

Bacterial cells: 0.1 mg. dry weight per ml. suspension. Length of light path: 10 mm. A sheet of oiled filter paper was attached between the suspension and photocell according to the method of Shibata (*3*). Shimadzu spectrophotometer Type QB-50 was used.

light period of 60 minutes.

It is apparent from these results that the two action spectra obtained are identical in showing a distinct peak at about 600 mμ, and a minimum

TABLE II

Photochemical Assimilation of Pyruvic Acid Occurring at Different Wavelengths

Experiment No. 10

Monochromatic light applied	Wavelength (mμ)	Pyruvate recovered in the medium (μM)	Pyruvate consumed (μM)	Relative activity of pyruvate assimilation (%)
A	395	1.63	0.87	87
B	455	1.87	0.63	63
D	534	2.25	0.25	25
E	552	1.94	0.56	56
F	597	1.50	1.00	100
H	700	1.82	0.68	68
Dark	—	2·50	0.00	0

Bacterial cells: 39 mg. dry weight per vessel. Initial amount of pyruvate in the medium, 2.5 μM. Reaction period, 60 minutes. For further details see text.

at about 540 mμ with a steady uprising towards the shorter wavelengths. These spectra are similar to the absorption spectrum of bacteriochlorophyll (see, for example, Goodwin (*5*)), but are different, in some important point, from the absorption spectrum shown by intact bacterial cells. Intact bacterial cells show a maximum at 590 mμ, which is due to bacteriochlorophyll and conceivably corresponds to the maximum at 600 mμ in the abovementioned action spectra. However, intact bacterial cells show an assembly of absorption bands in the region from 460 to 550 mμ with a maximum at 500 mμ, which is entirely lacking in the action spectra. Obviously these absorption bands are due to the carotenoid pigments contained in bacterial cells. It may, therefore, be inferred that bacteriochlorophyll, but not carotenoid pigments, participates in the two photochemical processes investigated. The coincidence of the action spectra of the two processes points to the functioning of one and the same photochemical machinery in them. The question as to by what mechanism the photochemical metabolism of pyruvate is related to the process of carbon dioxide assimilation is left open to further investigations.

SUMMARY

1. *Rhodopseudomonas palustris* has been known to perform, besides typical bacterial photosynthesis, a process of photochemical assimilation of pyruvate under anaerobic conditions. Using monochromatic lights of different wavelengths, the action spectra of these two processes were measured.

2. The two action spectra obtained were identical in showing a distinct peak at about 600 mμ and a minimum at about 540 mμ with a steady uprising towards the shorter wavelengths.

3. By comparing these spectra with the absorption spectrum of intact bacterial cells, it was inferred that in the mechanisms of the two processes investigated, bacteriochlorophyll, but not carotenoid pigments, is involved as the photo-catalytic agent.

The authors' best thanks are due to Prof. H. Tamiya for his helpful guidance and encouragement throughout this work. The authors are also indebted to Prof. A. Takamiya for his kind advice and to Dr. M. Nishimura for his able techical suggestions. The present work was supported by a grant from the Ministry of Education, which is gratefully acknowledged here.

REFERENCES

(*1*) Morita, S., *J. Biochem.*, **42**, 533 (1955)

(*2*) Manten, A., *Antonie van Leeuwenhock*, **14**, 65 (1947) Rabinowitch, E. I., "Photosynthesis", Intersc. Publ. Inc., New York, Vol. **II** part 1 (1945) Duysens, L. N. M., "Transfer of Excitation Energy in Photosynthesis" Doctoral thesis, University of Utrecht (1952)

(*3*) Shibata K., Benson, A. A., and Calvin, M., *Biochim. Biophys. Acta,* **15**, 461 (1954)

(*4*) Shimizu, T., *J. Biochem.*, **37**, 421 (1940)

(*5*) Goodwin, T. W., *Biochim. Biophys. Acta*, **18**, 309 (1955)

NOTES

The Journal of Biochemistry, Vol. 45, No. 7, 1958

PREPARATION OF CRYSTALLINE CYTOCHROME C FROM WHALE HEART MUSCLE

By SHIGEKI MINAKAMI

(*From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo*)

KOITI TITANI, HISAYUKI ISHIKURA AND KENJI TAKAHASHI

(*From the Department of Chemistry, Faculty of Science, University of Tokyo, Tokyo*)

(Received for publication, February 28, 1958)

Recently crystallization of cytochrome c was reported from several laboratories. Bodo was the first to report crystallization of cytochrome c using ammonium sulfate (*1*). He prepared cytochrome c from an unusual source, *i.e.* muscle of king penguine. Hagihara and others in Okunuki's laboratory succeeded in the crystallization of the cytochrome from more convenient sources, such as baker's yeast and heart muscle (beef, pig or fish) and the procedures were reported in detail (*2*). Their method is quite reproducible and the present authors also have succeeded in the crystallization of yeast cytochrome c.

In the course of our studies on the sructure of cytochrome c, the preparation of the pure protein on a large scale was intended. The authors have tried to prepare the cytochrome from whale heart muscle, as large amounts of frozen whale heart are obtainable in Japan. The cytochrome was obtained in crystalline form by the slight modification of the method according to Bodo or Hagihara *et al.* The following procedure was adopted for the preparation.

Minced whale heart was extracted with 0.1 N sulfuric acid (1 liter acid solution per kg. of the mince) and, after neutralization and dilution (about three fold), the extract was directly treated with Amberlite IRC 50 (NH_4^+ form). For the treatment of large volumes of the extract, the authors used a funnel with sintered glass filter (diameter; 12 cm.) and the rate of flow was enhanced by suction. By this procedure, more than 100 liter of the extract could successfully be treated in a comparatively short time. After elution with ammonium sulfate solution, the cytochrome was precipitated by using ammonium sulfate and trichloroacetic acid. Then the protein was purified by the IRC 50 chromatography according to the method of Boardman and Partridge (*3*) and concentrated by using the same ion-exchanger.

The concentrated solution (more than 5 per cent) was brought to pH

9 with ammonia water and reduced by minimum amount of cysteine. The fine powder of ammonium sulfate was added to the solution until it became somewhat turbid (about 0.9 saturation) and then cooled in a refrigerator. The crystals were formed within several days (Fig. 1).

The cytochrome c of whale heart muscle was easy to crystallize as compared with that of equine or bovine heart. However, it had a disadvantage that a large amount of myoglobin in the sulfuric acid or trichloroacetic acid extract disturbed the purification. Furthermore, the yield was poor (about 1 g. of pure preparation per 50 kg. of mince). The content of cytochrome c in whale heart was small as would be anticipated from the data of Drabkin (*4*) that there is an inverse relationship between the cytochrome c content per unit mass of different species and their body size. Accordingly, whale heart was proved to be inadequate for the large scale preparation of cytochrome c.

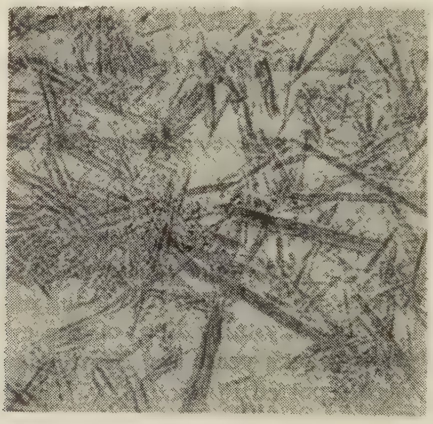


FIG. 1. Crystalline cytochrome c of whale heart muscle.

Paper electrophoresis at various pH's, ion exchange resin chromatography at various cation concentrations and pH's, determination of iron content, as well as the C-terminal determination (*5*) revealed no distinct difference between the cytochrome of whale heart muscle and other samples of mammalian cytochrome c (beef or horse).

The authors express their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for their interests during this work. Thanks are also due to Prof. K. Okunuki for his kindness to allow one of the authors (K. Titani) to get the experience of the preparation of cytochrome c at his laboratory. The authors acknowledge the gift of frozen whale heart by Kyokuyo Whaling Co., Ltd.

REFERENCES

(*1*) Bodo, G., *Nature*, **176**, 829 (1955)

(*2*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K., *Nature*, **178**, 629 (1956)

Hagihara, B., Morikasa, I., Sekuzu, I., Horio, T., and Okunuki, K., *Nature*, **178**, 631 (1956)

Hagihara, B., Tagawa, K., Nozaki, M., Morikawa, I., Yamashita, J., and Okunuki, K., *Nature*, **179**, 249 (1957)

(3) Boardman, N. K., and Partridge, S. M., *Biochem. J.*, **59**, 543 (1955)

(4) Drabkin, D. K., *J. Biol. Chem.*, **182** 317 (1950)

(5) Titani, K., Ishikura, H., and Minakami, S. *J. Biochem.*, **44**, 499 (1957)

昭和 32 年 7 月 9 日 第三種郵便物認可
第 45 巻 第 7 号 昭和 33 年 7 月 25 日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 7, July 25, 1958

Vol. 45 **No. 7**

CONTENTS

PAGE

HAYAKAWA, Shohei, SABURI, Yasuo and HOSHIJIMA, Hideyuki. Microbiological degradation of bile acids. XIX. Supplementary studies on cholic acid degradation by *Streptomyces rubescens* 465

FUKUDA, Toshifumi and HAYASHI, Toshio. Biochemical studies on the formation of the silkprotein. V. Conversion of glyoxylic acid to glycine in the silkworm larva 469

NOJIMA, Shoshichi, KONDO, Eiko and MIZUNO, Den'ichi. Distribution of inositol in the fraction of lipides from BCG 475

SHIMURA, Kensuke, FUKAI, Hironobu, SUTO, Seiichi and HOSHI, Reiko. Studies on the biosynthesis of silk fibroin. I. Incorporation of glycine-1-C^{14} into fibroin *in vivo* 481

TSUKADA, Yasuzo, TAKAGAKI, Genkichiro and HIRANO, Shusuke. Incorporation of radioactive phosphate into protein-bound phosphorus fractions of brain slices in reference to its relation to the metabolic activity 489

MATSUBARA, Hiroshi and NISHIMURA, Shigehiko. Crystalline bacterial proteinase. V. On the reaction with diisopropyl fluorophosphate 503

TANAKA, Kentaro and USAMI, Tadao. Studies on the ribonucleic acid from tobacco mosaic virus 511

HIGUCHI, Takayoshi. Further studies on phenol oxidase related to the lignin biosynthesis 515

ANDO, Toshio and HASHIMOTO, Chieko. Studies on protamins. V. Changes of the proteins in the cell nuclei of the testis during the formation of spermatozoa of the rainbow-trout (*Salmo irideus*) 529

TAKAMATSU, Ken and MORITA, Shigehiko. Action spectra of photosynthesis and photochemical assimilation of pyruvic acid in *Rhodopseudomonas palustris* 541

NOTES

MINAKAMI, Shigeki. Preparation of crystalline cytochrome c from whale heart muscle 547